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LEUKOTRIENES MEDIATE HYPEROXIA-INDUCED LUNG DAMAGE IN NEWBORN RATS

BY

JACQUELINE SARAH BURGHARDT ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**.

DEPARTMENT OF PHYSIOLOGY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **LEUKOTRIENES MEDIATE HYPEROXIA-INDUCED LUNG DAMAGE IN NEWBORN RATS** submitted by **JACQUELINE SARAH BURGHARDT** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**.

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ABSTRACT

Hyperoxia can severely damage the developing lung. In particular, the inhibition of alveolarization caused by oxygen toxicity can lead to profound long-term consequences. Questions remain as to the mechanisms of oxygen toxicity in the newborn. The levels of various mediators including leukotrienes (LTs) are altered by hyperoxia and may therefore play a role in its pathogenic action. The main focus of this study was to investigate the possible role of LTs in newborn hyperoxic alveolar and airway damage. Rat pups were placed into >95% O_2 from days 4-14 (period of alveolarization), 4-9, or 9-14 in different studies, and LT levels and lung morphometry were assessed at ages 9 days and 14 days. In a separate set of studies looking at airway function, rats were kept in ~65% O_2 from day 15-32 (subsequent to exposure to >95% O_2 from days 4-14) and pulmonary function and airway morphometry were investigated. LT synthesis inhibitors (WY-50,295 and MK-0591) or receptor antagonists (WY-50,295 and MK-0571) were used to investigate the role of LTs. Hyperoxic exposure caused an increase in LT production by the lung at days 9, 14, and day 28. Hyperoxia also prevented alveolarization from occurring as measured on day 14 and caused airway smooth muscle layer thickening and airway hyperreactivity at 5 weeks of age. All of these phenomena were prevented in hyperoxic animals in which LT synthesis was inhibited by Wy-50,295 or MK-0591. Exposure to hyperoxia had a more significant effect when administered days 4-9 compared to days 9-14, however, this "critical period" of hyperoxic exposure was not associated with a critical period for LTs, as LT inhibition from days 4-9, 9-14, or 4-14 all prevented damage caused by exposure from days 4-14. Preliminary immunocytochemical investigations into the location and quantity of the enzyme that

makes LTs from arachidonic acid, 5-LO suggest that hyperoxic animals have more 5-LO present in the lungs, especially certain airway epithelial cells, compared to air controls at day 14. An attempt was made to administer LTC_4 and LTD_4, and while they did not cause a major inhibition of alveolarization, they did produce significant septal thinning. Administration of MK-0571 to hyperoxic pups did prevent alveolar damage somewhat, although not to the extent of WY-50,295 or MK-0591. From these studies we can conclude that LTs have a role in mediating hyperoxic lung damage in newborn rats.

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LIST OF ABBREVIATIONS

12-LO	12-lipoxygenase
15-LO	15-lipoxygenase
5-LO	5-lipoxygenase
6-keto $PGF_{1\alpha}$	6-keto prostaglandin $F_{1\alpha}$
AA	arachidonic acid
A_{asp}	airspace area
A_{EL}	epithelial layer area
AP-1	activating protein-1
A_{SML}	smooth muscle layer area
ATP	adenosine triphophate
BAL	bronchoalveolar lavage
BM	basement membrane
BPD	bronchopulmonary dysplasia
BR_L	baseline pulmonary resistance
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CAT	catalase
d	day
DAG	diacyl glycerol
DNA	deoxyribonucleic acid
DPPC	dipalmitoylphosphatidyl choline
EC	endothelial cell
EGF	epidermal growth factor
EL	epithelial layer
ELW	extravascular lung water

FA	field area
FBM	fetal breathing movement
FGF	fibroblast growth factor
FLAP	five lipoxygenase activating protein
g	gram
GM-CSF	granulocyte monocyte-colony stimulating factor
GP	glutathione peroxidase
H_2O	water
HPETE	hydroperoxyeicosatetranenoic acid
ICC	immunocytochemistry
IFN	interferon
IL	interleukin
IP_3	inositol trisphosphate
kg	kilogram
KGF	keratinocyte growth factor
L_{BM}	length of basement membrane
Lm	mean linear chord length
LT	leukotriene
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
MAPK	mitogen-activated protein kinase
mg	milligram
µg	microgram
MK-0571	3[2 Methoxy 4[(2 Methylbenzene sulfonyl) carbamoyl]]Benzyl Methyl 1h Indole 5 Carbamic Acid Cyclopentyl Ester

MK-0591	[3 Tert Butyl Thiol (4 Chlorobenzyl) 5 (2 Quinolinylmethoxy) 2 Indoyl] 2,2 Dimethylpropionic acid
μl	microlitre
ml	milliliter
mm	millimeter
μm	micrometer
MMP	matrix metalloproteinase
n	number of replications
NF-κB	nuclear factor-kappa B
O_2	molecular oxygen
p	probability
P/A	perimeter/area
PAF	platelet activating factor
PC	phosphatidyl choline
PDGF	platelet derived growth factor
PE	phosphatidyl ethanolamine
PG	prostaglandin
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGG_2	prostaglandin G_2
PGHS	prostaglandin enodperoxide H synthase
PGI_2	prostaglandin I_2
PGL	phophatidyl glycerol
PI	phosphatidyl inositol
PKC	protein kinase C
PLA_2	phospholipase A_2

PLAP	phopholipase activating protein
PLD	phopholipase D
PMNL	polymorphonuclear leunkocyte
po	oral
RA	room air
RBC	red blood cell
R_L	pulmonary resistance
sc	subcutaneous
SD	standard deviation
SISA	specific internal surface area
SMC	smooth muscle cell
SOD	superoxide dismutase
SP-A, B, C, D	surfactant associated protein-A, B, C, D
TGF	transforming growth factor
THP-1 cells	human moncytic cell line
Th_{Sept}	septal thickness
TNF	tumor necrosis factor
TW	total airway wall area
Tx	thromboxane
V	vehicle
$V_{aspunit}$	airspace unit volume
V_P	volume of parenchymal tissue
Wy-50,295	Alpha Methyl 6 (2 Quinolylmethoxy) 2 Naphthylacetic Acid

I. BACKGROUND

A. INTRODUCTION

In many species, including rat and human, the lung at birth is not completely developed. It has been well established that in many mammalian species, alveoli are formed postnatally by the septation of saccules. The process is poorly understood but we do know that certain factors such as glucocorticoids, retinoic acid, growth factors, cytokines, and eicosanoids can influence the normal formation of alveoli. Another factor that is quite detrimental to the newborn lung is hyperoxia.

Oxygen can cause damage to proteins, cell membranes, and DNA through its breakdown products, the oxygen radicals. In addition, hyperoxia has the ability to alter the production of various factors by the lung, the consequences of which are not fully understood. The leukotrienes (LTs) are one group of mediators that may contribute to hyperoxia's effects on the developing lung.

This background section provides a brief review of the various phases of and some of the factors affecting lung development, focussing on postnatal events in the rat. Emphasis is placed on the effects of hyperoxia and LTs.

B. LUNG DEVELOPMENT

i) Stages of Lung Development

Lung development can be subdivided into 5 stages: 1) embryonic period - development of major airways; 2) pseudoglandular period - development of airways into terminal bronchioles; 3) canalicular period - development of acinus; 4) saccular period -

subdivision of acinus to form saccules; and 5) alveolar period - formation of alveoli. The morphological sequence of lung development is similar for both early and late stages of development in humans and rats. The rat has become one of the most studied animals in this regard (4) and, as a result, is an appropriate model for the study of lung development and the effects various factors have on this process (models for studying hyperoxia will be discussed later).

The Embryonic Stage

The embryonic stage of lung development is usually considered to take place from 0-5 weeks gestation in the human and 0-13 days gestation in the rat (47). The lung originates as a diverticulum of the ventral wall of the caudal end of the laryngotracheal tube, which divides into two knob-like bronchial buds at 3-4 weeks gestation in humans. Outgrowth of these buds into the splanchnic mesenchyme produces the left and right primary bronchi. The endodermal tissue later becomes the epithelial lining of the pulmonary airways. Each primary bronchial bud divides monopodially to give rise to a lateral diverticulum or bud; subsequently, the right lung bud gives rise on its craniodorsal side to a second monopodial diverticulum. Each secondary bronchus is a stem bronchus that will be a lobar bronchus. These stem bronchi are destined to branch and rebranch and with the surrounding pulmonary mesenchyme (which will provide the elastic tissue, smooth muscles, cartilage, vascular system and other connective tissue), which will give rise to the definitive pulmonary lobes that characterize adult lung organization (70).

By day 34 in the human and about day 13 in the rat, the major bronchopulmonary segments have been formed and the airways are lined with tall columnar cells. The splanchnic mesenchymal tissue surrounding airways eventually forms non-epithelial blood vessels, lymphatics, cartilage, smooth muscle and connective tissue. At maturity over 40 different cell types will have evolved from the primitive endodermal and mesenchymal cells (110).

The Pseudoglandular Stage

The pseudoglandular stage occurs between weeks 5-16 in humans and days 13-18 in rats (47). During this stage the lung resembles a system of branching tubules that terminate in exocrine gland like-structures. Mesenchymal tissue is invaded by branches of distal extremity of the tubes of epithelial (endodermal) cells. Branching of the distal 'airway' is stimulated by interactions between epithelial cells and the surrounding mesenchyme (perhaps through the release of localing acting mitogenic factors). Epithelial cells lining the airways begin to differentiate starting at the hilum and progressing to the periphery and simple epithelial cells differentiate into goblet cells and mucus glands (284). Flatter cells with cilia appear by approximately 11-13 weeks in the human. Growth of the pulmonary circulation also occurs during this stage, with each of the major respiratory tubules being accompanied by a major artery (110). Vascular development has been extensively investigated prenatally (7, 125-127, 136-138). It is known that the arterial tree branches with the airways, whereas veins run in between the airway branches in connective tissue septa extending between each generation of dichotomously branching airways (297). The arteries have smaller branches in addition to the conventional branches running with the airways, which supply alveolar regions adjacent to walls of the airways. The veins depart from their interaxial course in the central lung portions, where the larger branches join the pulmonary hilum with the airways and arteries (47).

Development of airway smooth muscle is intimately linked with the formation of the tracheobronchial tree. The smooth muscle cell (SMC) is the first cell to become visible as a condensation of the mesenchyme near the basement membrane along the epithelial tubules (6-8 weeks in humans) (179, 186). SMC investment around the airways extends from its origins in the trachea, mainstem, and lobar bronchi to the segmental bronchi, terminal and respiratory bronchioles, and alveolar ducts (172, 186). Early in gestation, the SM becomes innervated (202, 274).

The Canalicular Stage

During the canalicular stage (human 16-26 weeks, rat 18-20 days) there is expansion of the airways, with thinning of the mesenchyme. There is an increase in the proportion of potential air space. This stage also sees the establishment of recognizable respiratory units with a cluster of terminal bronchioles, several 'respiratory' bronchioles and a peripheral cluster of closely branched buds that will form saccules. Capillaries of the mesenchyme invest the tubules, coming into close contact with the epithelial layer (110). During this stage the distance between the epithelial surface and blood cells in capillaries becomes smaller. Eventual type I cells become flatter as their cytoplasm becomes attenuated. In humans, surfactant-containing lamellar bodies first appear in type II cells (110).

The Saccular Stage

Progressive enlargement of peripheral airspaces occurs during this last stage of fetal development (human 26 weeks to term, rat 20 day to term) along with development of alveolar ducts or sacs. The formation of pulmonary surfactant is increased in preparation for gas exchange and birth. The capillary network proliferates with further thinning of mesenchyme (110).

The Alveolar Stage

The events pertaining to the alveolar stage (36 weeks gestation to 1-2 years of age in humans and postnatal days 4 – 14 in rats) have been thoroughly investigated in the rat lung (46, 48, 196, 244) and I will focus on this species.

At birth the rat lung is still in the saccular stage. There are no alveoli and the septa of the parenchyma, the primary septa, as they are called in this stage, are thick and contain a double capillary network. Within 3 weeks, this original structure is dramatically transformed. The contours of the airspaces increase in complexity as a result of a rapid process of septation. After about the 4th postnatal day, "buds" (later known as secondary septa) appear along the primary septa and later increase in height and divide the saccules into alveoli. Both the primary septa at birth and the secondary septa formed later contain two capillary layers unlike adult lungs which have one. Rapid alveolarization in the rat occurs for about 10 days. Postnatal events in the human lungs do not differ greatly from that of rat (317, 318).

ii) Regulators of Postnatal Lung Development

Various factors have been shown to play a role in alveolar development. Some of the more well known are corticosteroid hormones, growth factors and cytokines. Lately, retinoic acid has also been implicated as having a substantial effect on the formation of alveoli. Eicosanoids also have the potential to affect lung development, as they have been shown to alter growth and differentiation of various lung cell types, collagen, fetal breathing movements (which have been shown to influence lung development), and surfactant. Most information exists for the prostaglandins and much less is known about leukotrienes.

Corticosteroid Hormones

Liggins first described the *in vivo* effect of hormones on lung maturation in 1969 (181). When studying the effect of glucocorticoids on premature labour, he discovered that treated fetal sheep had better lung development than expected at that age. The clear demonstration by Burri *et al.* (46, 48) that in some species alveolarization is an early postnatal rather than prenatal event raised the possibility that corticosteroid hormones, which strongly influence the postnatal development of the gastrointestinal system (133), the lung's origin, might also influence the postnatal structure of the lung. The time-course of alveolarization (46, 48) and serum concentration of corticosterone (132) in rats suggests that septation takes place when the levels of this hormone are at their lowest. Thinning of the alveolar walls occurs when the levels are highest. Glucocorticoids inhibit cell division in several tissues including the lung (185, 195). Dexamethasone was administered to rats from days 4 - 13 in an attempt to keep serum corticosteroid levels high during the period of septation. This treatment lead to impaired septation, a defect which persisted after dexamethasone was removed (measured at 60 days and in another study 90 days) (195, 256).

Within 48 hours of the first two treatments with dexamethasone, begun on day 4, as much septal thinning occured as normally takes place in diluent-treated rats between days 4 and 14 (195). The molecular basis for dexamethasone's effects remain elusive, but there are three proteins whose expression peaks in the rat lung during the time septation normally occurs and whose actions indicate that they influence development. These include β-galactoside-binding protein (59, 241), a cellular retinol-binding protein (222), and a cellular retinoic acid binding protein (222). Dexamethasone treatment of neonatal rats decreases the lung expression of the β-galactoside-binding protein (59), and the expression in the lung of cellular retinol-binding protein is also decreased within hours of treating adult rats with dexamethasone (254). Retinoids and glucocorticoids may have antagonistic actions (98, 117, 254).

Retinoic Acid

Massaro *et al.*(198) showed that treating neonatal rats with retinoic acid starting on day 3, one day before starting treatment with dexamethasone, and continuing during treatment with dexamethasone until day 13, prevented the inhibition of septation by dexamethasone. Thus, the possibility of a role for retinoic acid in lung development was raised. Retinoic acid administered to otherwise untreated rats increased the number of alveoli formed but induced the formation of smaller alveoli than in normal rats; this prevented an increase in the gas-exchange surface area of the lung. This may suggest a control mechanism that inhibits the size of alveoli formed under the influence of a pharmacological agent that induces an increase in the number of alveoli formed in the absence of a metabolic need for a larger gas-exchange surface area (197). Massaro *et al.* suggest that eruption and elongation of septum have different regulators; retinoic acid induces the eruption of a septum, but other agents or conditions regulate its length.

Growth Factors and Cytokines

No discussion of growth and development would be complete without mentioning the role of growth factors and cytokines. It is unknown at this point exactly how these factors interact with one another and with other types of mediators to effect the developing lung. Numerous studies have been done using various animal models, including knockouts and animals over-expressing certain genes. Table 1 shows some of the factors that have been shown to have an effect on alveoli.

Eicosanoids

The lungs of fetal and neonatal animals and humans are capable of producing eicosanoids (prostaglandins (PG), leukotrienes (LT), and associated hydroxy fatty acids), and are capable of responding to such factors. These responses occur in association with a variety of biological processes, including the control of vascular and airway diameter, effects on cell proliferation, the production and catabolism of collagen, mucous and lung fluid production, the release of surfactant and the synthesis of surfactant apoprotein A, the inflammatory response, and markers of cytotoxicity.

Despite studies occurring in this area for approximately 30 years, our understanding of the control of synthesis and biological function of eicosanoids in the lung during the perinatal period is sketchy and incomplete. There is little overall appreciation of the role of eicosanoids in the development of the perinatal lung, their role in the acute regulation of lung physiology, their function in the transition to independent life, or their role in lung disease in the perinatal period.

Information about the effects of LTs specifically is sparse despite indications that they may indeed be important mediators in the newborn lung, especially of disease processes.

Synthesis

Arachidonic acid (AA) is a 20-carbon fatty acid that is a common constituent of phospholipids in cell membranes. Upon stimulation of a cell, through G-protein coupled receptors and subsequent mobilization of calcium, free AA is released from the membrane by the action of phospholipases (81). A number of signal transduction pathways may be involved in these processes. One pathway involves the sequential degradation of phophatidyl inositol (PI) via a sequence of reactions beginning with phospholipase C [2] and followed by diglyceride lipase and monoglyceride lipase [3]. Another involves an initial conversion of phophatidylethanolamine (PE) or phosphatidylcholine (PC) to phosphatidic acid catalyzed by a phopholipase D, followed by formation of diglyceride and monoglyceride [3,4,5]. A third pathway involves a direct action of a PLA_2 on phospholipid that, depending on the PLA_2, could include 1,2-diacyl- or 1-0-alkyl-2-acyl-PI, PE, or PC. Following its cleavage from the sn-2 position of membrane associated phospholipids, arachidonic acid may be metabolized via the cyclooxygenase or lipoxygenase pathways.

Cycolooxygenase

The transformation of free AA via PGHS (cyclooxygenase) leads to the formation of PGD_2, $PGE_{2\alpha}$, and PGF_2, as well as the formation of thromboxane (Tx) and prostacyclin (PGI_2) (See Figure 1a). There are two PGHS isozymes called PGHS-1 and PGHS-2. PGHS-1 has a signal peptide and, following cleavage of the signal peptide (between amino acid residue 26 and 27 in the case of murine PGHS) contains 576 amino acids and has a molecular weight of 65,500 Da. PGHS-2 is considered to be the inducible isoform (72 kDa). It varies significantly from PGHS-1 at positions prior to amino acid residue 30 (271). PGHS-1 mRNA is present in many cell lines and in extracts of virtually all mammalian tissues. PGHS-2 is detectable in prostate, brain, testes, vascular endothelial cells and lung (268), but is found in low abundance in kidney and most other major organs [10]. PGHS-1 and -2 both catalyze two reactions (272). In the first, arachidonic acid is converted to PGG_2; no net oxidation-reduction occurs in this reaction. PGHS catalyzes the attachment of molecular oxygen at the 11th carbon of AA. There is a subsequent rearrangement to a cyclic endoperoxide in which a molecule of oxygen bridges carbons 9 and 11. An introduction of a second molecule of oxygen at carbon 15 yields PGG_2. The second reaction is a peroxidase reaction involving a net two electron reduction of the 15-hydroperoxyl group of PGG_2 to the 15-hydroxyl group of PGH_2. These 2 reactions occur at distinct sites.

Prostaglandin H_2 is subsequently metabolized into PGs and TxA_2 by a series of cell- and species-specific synthases whose activation may also be stimulus specific.

Although the endoperoxide PGH_2 has important biological effects (notably aggregation of platelets), the principal biologically active prostanoids are formed enzymatically and/or non-enzymatically from PGH_2. PGD_2 is formed from PGH_2 by PGD synthase, PGE_2 by PGE synthase (once called PGE isomerase) and $PGF_{2\alpha}$ by PGF synthase. The unstable metabolite PGI_2 is produced from PGH_2 by PGI synthase, and the even more highly unstable metabolite TxA_2 is formed by the action of TxA synthase. Non-enzymatic processes can result in formation of PGE_2 and PGD_2 from endoperoxide that escapes the cell; non-enzymatic formation of PGD_2 can be catalyzed by serum albumin (271). Cooperative formation of prostanoids by two or more adjacent cell types can also occur.

Lipoxygenase

Unlike the prostanoids, the lipoxygenase products do not have a "prostanoic acid" backbone or (in the case of thromboxanes) a closely related structure. There are three major lipoxygenases named for their ability to insert molecular oxygen at a specific carbon of AA. The first lipoxygenase to be described was that converting AA into 12-lipoxygenase (12-LO) products. The discovery of the 5-lipoxygenase (5-LO) pathway leading to the formation of the LTs caused an explosion of interest in this area. Subsequently, interest in the 15-lipoxygenase (15-LO) area led to the discovery of the lipoxins (261). Although 12-LO and 15-LO products have been localized in the lung there has been little investigation regarding the significance of these products in the perinatal lung.

Five-lipoxygenase has been characterized from a number of mammalian sources including pig, human, rat, and guinea pig. Human 5-LO is a 78 kDa protein with a requirement for Ca^{2+} and ATP. The human 5-LO gene is estimated to be <82 kb and consists of 14 exons divided by 13 introns (214). Despite the absence of obvious regulatory sequences in the 5-LO gene, 5-LO must be regulated in a highly cell-specific fashion as substantial production of 5-LO is only well documented for the cells of myeloid lineage and not for many others (214) (TGF-β can regulate 5-LO activity without affecting 5-LO mRNA expression (278)).

The principal 5-LO metabolites of AA especially in leukocytes, but also in lung, are the leukotrienes, so named for their occurrence in leukocytes and a characteristic conjugated triene structure (122). The LTs formed from AA are derived through a 5-hydroperoxy intermediate (5-HPETE) (See Figure 1b). They have 4 double bonds and are thus called the 4-series, using subscript nomenclature similar to that of the prostanoids. 5-HPETE can be converted by removal of water to a 5,6-epoxide containing a conjugated triene structure called leukotriene A_4 (LTA_4). This dehydrase activity is also associated with 5-LO. LTB_4 is a 5, 12-hydroxy compound derived from LTA_4 by the addition of water via the action of LTA_4 hydrolase or to LTC_4 by addition of a glutathionyl group at carbon 6 by the action of glutathione-S-transferase. Leukotrienes containing peptides or amino acids at carbon 6 are termed peptidoleukotrienes (peptido-LTs). LTC_4 can be cleaved by γ-glutamyl-transpeptidase to produce LTD_4, and LTD_4 can be further metabolized to LTE_4 by a dipeptidase (122).

Addition of AA to cells containing PGHS-1 or -2 results in the synthesis of PGs, implying that the key event in the production of these eicosanoids is the activation of

PLA_2 and release of substrate. This is not the case with 5-LO, and LT synthesis only occurs in intact cells following exposure to certain stimuli that induce a rise in intracellular Ca^{2+}. 5-LO is dependent on ATP and the 18 kDa 5-LO activating protein, FLAP. The commonly held theory is that following activation of a cell, there is a rise in intracellular Ca^{2+} that results in the activation and translocation of 5-LO as well as high molecular weight PLA_2, with 5-LO moving to the docking protein FLAP. After formation of LT, 5-LO would undergo turnover-associated suicide inactivation. A more recent hypothesis is that FLAP binds to AA and facilitates its transfer to 5-LO, allowing the 5-LO reaction to occur in a more efficient fashion (214). FLAP is expressed in cells of myeloid origin, PMNL (polymorphonuclear leukocytes), macrophages, eosinophils, basophils, lymphoblastoid cells, as well as type II pneumocytes and airway epithelial cells.

Metabolic intermediates from one cell type can be used by another cell type for the production of eicosanoids. Stimulated platelets form endoperoxides which in the endothelial cell will produce prostacyclin. Neutrophilic LTA_4 (stabilized by albumin) will form LTB_4 once passed on to erythrocytes, vascular endothelial cells and platelets (RBC and EC do not have 5-LO).

Receptors

Prostaglandin Receptors

Prostaglandin receptors have been found in airways including human bronchi (107), guinea pig (107), and cat (8, 160) trachea. They have also been found in guinea pig and dog lung strips (61).

Leukotriene B_4 Receptors

Two distinct binding sites of high and low affinity for LTB_4 exist on the surface of neutrophils (113, 114, 180). These G-coupled receptors are members of the rhodopsin-like receptor subfamily (113, 114). Competitive binding studies in neutrophils and eosinophils have shown that there is a double bond at the c-6 position, the (5s,12r)-hydroxyl groups, and an intact eicosanoid backbone are structural and sterospecific requirements for LTB_4 binding to its receptor and induction of neutrophil chemotaxis (33, 175, 218).

Peptido-Leukotriene Receptors

There is uncertainty as to the number and specficity of receptors for the peptido-LTs. Highly selective receptors for LTC_4 ($CystLT_2$) have been identified in the lungs of rats (240) and guinea pigs (141); similar LTC_4-specfic receptors have not been identified in human lung tissue (43). The demonstrated ability of LTC_4 to contract bronchial smooth muscle may be explained by its bioconversion to LTD_4.

In human lung parenchyma, LTD_4 interacts with high and low affinity receptors of the rhodopsin-like superfamily ($CystLT_1$) (253). Selective LTD_4 receptor antagonists inhibit both LTC_4 and LTD_4 contractile activity in human lung tissue, suggesting that LTC_4 interacts with a common LTD_4 binding site (5, 43). Because LTE_4 competes for the LTD_4 receptor molecule on the cell surface, this may be the case with LTE_4 as well.

The signal transduction process for LTD_4 receptors can be divided into immediate and late phase. Within seconds after LTD_4 reacts with the receptor, the receptors interact with at least two guanine nucleotide binding proteins (G-proteins): pertussis toxin sensitive and insensitive proteins (68, 69). In contrast, LTB_4 receptors employ only a pertussis toxin-sensitive G-protein (112, 311). The LTD_4 receptors are coupled to Ca^{2+} mobilization by at least two signaling systems including a G-protein activated phosphoinositide-specifc phopholipase C (PI-PLC), and a receptor operated Ca^{2+} channel, which appear to be independent of the generation of IP_3 (69).

There is a rapid, transient increase in intracellular Ca^{2+} stores (69). Phosphoinositide metabolism is enhanced resulting in rapid increases in the inositol phosphates involved in intracellular signaling (an increase in PIP_2-specific and a PIP_3-specific PLC activation). Diacylglycerol (DAG) production is also increased (69, 210).

Diacylglycerol, inositol phosphate, and Ca^{2+} release leads to activation of protein kinase C (PKC) (296) which seem to play two roles. Firstly, it activates various intracellular proteins including topoisomerase 1 (295). In monocytic THP-1 cells, Ca^{2+} increase occurs along with an increase in mitogen-activated protein kinase (MAPK). This increase is prevented by inhibition of PKC or staurosporine. THP-1 cells have two distance signaling pathways, a pertussis toxin-insensitive MAPK activation through PKCα and RAF-1 and a pertussis toxin-sensitive chemotactic response. Secondly, PKC activation is involved in desensitzation of LTD_4 receptors (295, 296, 310).

Activation of topoisomerase-1 is involved in the second phase of the signaling process which involves the liberation and metabolism of AA. The second phase is normally initiated by increased transcription of a gene for phospholipase activating protein (PLAP), a protein that activates a phospotidylcholine-specific phospholipase A_2

(57). PLA_2 activation increases the release of AA which is metabolized via the cyclooxygenase and lipoxygenase pathways depending on the cell type.

Leukotrienes are not stored and cells that display LTD_4 receptors often contain 5-LO. Thus, 5-LO may be considered part of the signaling system regulating the level of the agonist (68). Therefore, 5-LO must be carefully regulated and controlled.

Effects of Eicosanoids on the Developing Lung

Cell differentiation and growth

A critical phase of human lung growth, which largely determines survival in extremely premature infants, occurs between 20 and 30 weeks gestation when distal airway progenitor cells differentiate into type I and II pneumocytes and terminal airsacs dilate. Lung primordium is a self-differentiating system *in vitro* (148), and in organ cultures from human fetuses, as early as 12 weeks gestation, the ductular epithelium differentiates into type II cells containing lamellar bodies of surfactant. In addition, the terminal air spaces dilate and the epithelial lining cells flatten to give the appearance of type I cells. This differentiation occurs in the absence of sera, exogenous growth factors, or hormones, suggesting the activation of paracrine or autocrine regulatory mechanisms.

Evidence points to a role for PGs in differentiation of the lung. Hume *et al.* (148) showed that PGE_2 but not $PGF_{2\alpha}$ accelerates the process of self-differentiation in fetal lung organ culture. The addition of indomethacin retards the process of self-differentiation, an effect which can be reversed by the addition of PGE_2 but not $PGF_{2\alpha}$. Also, the amount of PGE_2 and $PGF_{2\alpha}$ released into media from fetal lung organ cultures decreases with time, accompanied by a decline in immunohistochemical reactivity specific for these PGs, possibly representing down-regulation of PG synthesis as these cells differentiate (147, 148). In 1993, Hume *et al.* (147) concluded that oxidative and mechanical stresses occurring during tissue preparation may result in cell damage leading to increased PG release. This decreases as cells recover, explaining the rapid release and then drop-off. The above findings, together with the observation that ontogenic changes in amounts and types of PGs produced by fetal lungs (223) occur, suggest a role for PGs in lung cell differentiation and growth.

In vivo studies also provide evidence of the important effects PGs can have on alveolarization. Nagai *et al.* (215) examined the effect of indomethacin on early postnatal development of the lung in rats and found that administration of the inhibitor from postnatal days 3 to14 decreases PG levels and causes a marked increase in alveolar size and inhibition of septation, although quantitative lung growth is not altered. Supplementation of indomethacin treated animals with PGE_2 reduces these abnormalities to or toward normal.

Arachidonic acid metabolites may also regulate fibroblast proliferation in the developing lung. Addition of PGE_2, which elevates cAMP in human fibroblasts, results in inhibition of DNA synthesis stimulated by EGF or insulin (146). The basal release of AA and PGE_2, and endogenous levels of cAMP, rise during aging and become much greater than in young cells. Furthermore, ATP appears to exert its mitogenic effect, particularly on aged fibroblasts, at least partially by suppression of AA metabolism. Inhibition of tumor necrosis factor- and interleukin-1-induced PG production by human lung fibroblasts suppresses the negative effects of these growth factors on fibroblast proliferation (92). Thus, it is evident that PGs, likely through interaction with other regulatory factors, may provide an important negative control on lung fibroblast proliferation. This inhibitory effect of PGs may be counterbalanced by stimulatory effects of other eicosanoids. Baud *et al.* (21) have demonstrated that peptido-LTs stimulate human fibroblast thymidine-incorporation and increase cell numbers, but only when endogenous production of PG is blocked. This has significant implications, as an alteration in the balance between PG and LT production could modulate lung development.

Arachidonic acid metabolites may be critical intermediaries in the regulation of vascular cell growth. Dethlefsen *et al.* (84) have demonstrated, in cultured bovine capillary endothelial cells (EC) and aortic smooth muscle cells (SMC), that inhibition of release of AA metabolites, as well as blockade of the lipoxygenase pathway, suppresses both basal and growth-factor stimulated cell proliferation. This inhibition is reversible and not due to cytotoxicity. Inhibition of the cyclooxygenase pathway has no effect on EC and aortic SMC proliferation. Thus, AA release appears to have an essential role in the mechanism by which a number of growth factors stimulate EC and aortic SMC growth, as does the lipoxygenase pathway. In contrast, prostanoids do not seem to play a central role in bovine vascular cell growth. Whether these findings can be applied to fetal lung cells or angiogenesis is unknown; however, the possibility exists.

Collagen

Interactions between cells and their extracellular matrix have important roles in embryonic development and organogenesis (3). Collagen is usually the predominant component of extracellular matrix, collagens type I and II being the major proteins in the interstitium of the lung parenchyma (55), and type IV is the collagen of basement membranes (91). In the lung, growth and alveolar development depend on remodelling of the connective tissue and deposition of new extracellular matrix as a scaffold for normal cell growth and differentiation. Larson *et al.* (173) have demonstrated rapid changes in procollagen mRNA levels in the rat lung during the perinatal period, supporting and extending the work of Bradley *et al.* (38) who observed a burst of collagen synthesis during the period of alveolarization in rabbits. Interference with collagen cross-linking decreases bronchial branching and alveolar multiplication *in vivo* (161), and stimulation of collagen degrading metalloproteinases in lung rudiments alters branching morphogenesis (106). Despite increases in total collagen during the phase of rapid lung development, Arden *et al.* (10) have shown a high level of lung collagen degradation during the cell proliferative phase, and basement membrane degrading collagenases, including MMP-2, are expressed at high levels during the immediate postnatal period when alveolar structures form (188, 247). Thus, an appropriate balance between collagen synthesis and degradation is an important factor in normal lung development.

Several PGs are known to have effects on fibroblast growth and collagen production in soft tissues (168), suggesting that the PG system may have an important physiological role in regulating collagen turnover vital for reorganization of lung structure during development. Prostaglandin E_1 has been shown to significantly lower the rate of collagen synthesis by human fetal lung fibroblasts without affecting overall protein synthesis, indicating a relatively specific effect (18, 23). Barile *et al.* (18) suggest that the most likely explanation for PG effects is a reduction in the amount of collagen mRNA, supported by earlier work by Varga *et al.* (294). Fine *et al.* have shown that PGE_2-induced inhibition of type I collagen formation by human embryonic lung fibroblasts is acheived through induction of a protein which suppresses transcription of $\alpha1(I)$ gene (94), the gene encoding one of three polypeptides which make up the type I collagen molecule. Although $\alpha2(I)$ mRNA levels are not affected during PGE_2-mediated inhibition of type I collagen formation in embryonic lung fibroblasts (94), this does not preclude an effect of PGs during posttranslational processing of the peptide chains encoded by this gene. Specific PGs may also reduce collagen synthesis by regulation of enzymes mediating the formation of stabilizing covalent cross-links that form between polypeptide chains in the same and adjacent collagen molecules. In neonatal rat lung fibroblasts, PGE_2 markedly reduces the activity of lysyl oxidase, and, consistant with this, the presence of indomethacin stimulates enzyme activity (31). Not only do PGs decrease synthesis of new collagen in fetal lungs, PGE_1 increases the amount of collagen degraded intracellularly (18, 23). It is possible that this increased intracellular degradation is a response to abnormal collagen formation (28) as a result of surplus pro$\alpha2(I)$ chains relative to pro$\alpha1(I)$, or destabilized collagen fibrils. As the amount of collagen synthesized in the lung is a function of the number of collagen-producing cells, specific eicosanoids may also affect collagen turnover through effects on the proliferation of cells such as pulmonary fibroblasts, as described in an earlier section. In addition, signal transduction events mediating biosynthesis of MMP-9, the collagenase active against collagen IV, by alveolar macrophages requires the presence of PGE_2, but not $PGF_{2\alpha}$, PGI_2, or LTs (264). As noted earlier in this discussion, levels of basement membrane-degrading collagenases such as MMP-9 and MMP-2 in the lung are altered in association with alveolarization and lung cell proliferation, implicating PGE_2 as an important factor in lung remodeling. From the above discussion, it is evident that the PG system has marked potential to be of physiological significance in regulating collagen turnover, thereby modulating growth and structural development of the lung.

Leukotrienes may also have a significant role in remodeling of the extracellular matrix during lung development. There is evidence to suggest that LTC_4 may be involved in the modulation of some fibroblast functions, particularly in those related to collagen turnover. Phan *et al.* (235) have reported that LTC_4 has a biological effect stimulating collagen synthesis. Others have shown that exposure of human lung fibroblasts to physiological concentrations of LTC_4 induces collagenase transcription and secretion in normally non-producing cells, and increases expression and synthesis in fibroblasts that normally produce the protein (203). Leukotrienes are able to mobilize intracellular calcium in mesenchymal cells (45, 209), and it is documented that collagenase levels are enhanced by an increase in intracellular Ca^{2+} (171, 292), suggesting that upregulation of metalloproteinases by LTC_4 may result from its role in this second-messenger system. Furthermore, LTs appear to constitute a novel signal transduction pathway in growth factor action. In two unrelated cell lines, EGF-induced Ca^{2+} channel activity depends on 5-LO metabolism, with the final common effector being LTC_4 (233). Synthesis of collagen by fetal rat lung epithelial cells is regulated, among other factors, by EGF (93), implicating a role for LTs in modulation of epithelial cell function to induce changes in the extracellular matrix.

Collagen turnover involves a stringently regulated balance between the deposition of the molecule, its degradation by specific collagenases, and the interaction among these metalloproteinases and their natural tissues inhibitors. Several eicosanoids including LTs appear to be involved in regulating these processes, giving them a dramatically important role in remodeling of the lung critical to growth and appropriate structural development

Fetal Breathing Movements

Fetal breathing movements (FBMs) have been identified in a number of mammalian species (77, 193) including humans (32), and there is considerable evidence to support such movements as an important determinant of pulmonary cell growth. Experimentation in animals and observations of human fetuses with congenital abnormalities potentially affecting the respiratory system indicate that FBMs are critical for normal prenatal lung development (87, 115, 308). *In vitro* studies (183, 184, 269) have shown that mechanical stretch of organotypic cultures of fetal rat lung cells in a manner simulating FBMs results in an increase in DNA synthesis and cell number. Breathing movements in fetal sheep, the species most studied, occur almost continuously until about 110 days of gestation (36) (total gestation is approximately 147 days), after which they become episodic. Upon differentiation of the electrocorticogram (at about 115 days), breathing episodes become associated with low voltage activity and rapid eye movements (76). Although the importance of FBMs in fetal lung development is well established, the physiological mechanisms regulating such movements is much less clear.

A number of studies have implicated a role for PGs, particularly PGE_2, in the control of FBMs. In fetal sheep, intravenous infusion of PGE_2, $PGF_{2\alpha}$, and several endoperoxide analogues significantly decrease the incidence of FBMs, with the most profound reduction occurring with PGE_2 (259). That endogenous PGs have a role in regulating FBMs is supported by studies involving inhibitors of PG synthesis. Breathing movements can be induced in fetal sheep during high voltage states with intravascular infusions of meclofenamate or indomethacin (164), which produce prolonged periods of breathing without altering the electrocorticogram. A possible explanation for the effects of cyclooxygenase inhibitors known to stimulate FBMs is a shunt to the lipoxygenase pathway and a consequent increase in LT synthesis. However, inhibition of lipoxygenase in fetal lambs neither stimulates FBMs nor affects the increase caused by PG inhibition (151). Wallen *et al.* (300) demonstated that the administration of progressively increasing doses of PGE_2 during continuing meclofenamate infusion in the fetal sheep gradually decreases the incidence of breathing movements, most markedly during high voltage activity, restoring a pattern of breathing movements similar to the physiological pattern of intermittent breathing movements associated with low voltage activity. Collectively, these findings suggest that inhibition of FBM during high voltage states may be mediated through a PG mechanism.

Much remains to be understood regarding the mechanism by which the PG system might regulate FBMs. Prostaglandin E_2 itself could act as a neurotransmitter (200), or may interact with other central neurochemicals such as catecholamines (246) to modulate neuronal function. As the PG system is able to modulate vascular resistance, local changes in cerebral blood flow leading to altered neuronal metabolism present an additional mechanism by which these eicosanoids could regulate breathing movements in the fetus.

Surfactant

Pulmonary surfactant is a developmentally-regulated lipoprotein synthesized and secreted by alveolar type II cells. The most recognized physiologic role of surfactant is its role in stabilizing the alveolar surface. A more recently recognized function of surfactant is that of a defense barrier between an organism and the outside world (70).

Mammalian surfactant contains 70-80% phospholipid, 10% protein, and neutral lipids. Phosphatidylcholine (PC) represents the majority of phospholipids, followed by phosphatidylglycerol (PGL), phosphatidylinositol (PI), and phosphatidylethanolamine (PE). Phosphatidylglycerol promotes the association of surfactant apoproteins with phospholipids and may play a role in recycling of surfactant by alveolar epithelial cells (50). Four surfactant proteins have been characterized. SP-A is hydrophilic and influences the surface properties of phospholipids. The hydrophobic proteins, SP-B and SP-C, permit rapid adsorption and spreading of phospholipids. SP-D is hydrophilic and has a role in host defense (70).

Surfactant production occurs relatively late in mammalian gestation (last 10-15% of gestation). Cells that eventually will become type II cells appear at about 11-12 weeks gestation in the human, but they do not contain lamellar bodies. SP-A gene expression and synthesis are initiated in fetal lung tissue during the third trimester of gestation. After 25-28 weeks, a substantial increase in the amount of phospholipid is observed in amniotic fluid, with a larger increase just before birth. A 4 to 20-fold postnatal surge is reported for lung lavage PC and dipalmitoylphosphatidyl choline (DPPC). At the same time, a decrease in the amount of sphingomyelin is seen. At about 35 weeks, an increase in PGL and a fall in PI are found. Labour is an important stimulus for the secretion of surfactant. Since the levels of PGs are also known to increase near term, the possibility exists that eicosanoids are involved in the regulation of surfactant production and secretion in the perinatal period.

A role for PGs in the regulation of surfactant synthesis by the perinatal lung is supported by the finding that administration of indomethacin to pregnant rabbits causes a decrease in alveolar surfactant content of newborn lungs (49). Furthermore, infusion of fetal sheep with indomethacin or meclofenamate decreases tracheal fluid surfactant flux (163). There is also evidence to suggest that PGs have an effect on SP-A gene expression. SP-A synthesis and gene expression are initiated in fetal lung tissue in concert with the developmental induction of surfactant glycerophospholipid synthesis. In studies using midtrimester human fetal lung explants, Mendelson *et al.* (204) observed that cAMP and glucocorticoids have pronounced effects on morphologic development and on levels on SP-A gene expression. Indomethacin markedly inhibits SP-A gene expression and cAMP formation, and the inhibitory effect on SP-A gene expression can be prevented by incubation with cAMP or PGE_2. Ballard (15) showed that PGE_1 and PGE_2 in the presence of indomethacin produce a parallel stimulation of cAMP content, SP-A and fatty acid synthase activity.

Despite the evidence implicating PGs in the regulation of surfactant during development, some studies fail to show a role for these eicosanoids. Baybutt *et al.* (24) showed that while AA and eicosapentaenoic acid stimulates surfactant release from adult type II cells, administration of indomethacin does not prevent the release. Cvetkovic *et al.*(71) found that human amniotic fluid at 26-39 weeks of gestation contains PGs but this has no correlation with the lecithin sphingomyelin ratio or the presence of PGL. Wallen *et al.* (301) found that infusion of meclofenamate 5 to 13 days before delivery in sheep significantly decreases plasma PGE_2 concentrations until the day of delivery, however, it does not alter the surfactant content of tracheal fluid and lung tissue. Leukotrienes were not measured in this study, and it is possible that the inhibition of PGs allows more AA to be available for shunting to the 5-LO pathway. Evidence does exist to suggest that LTs might also be involved in surfactant regulation.

It has been demonstrated that PC secretion is stimulated by LTs in primary cultures of adult rat type II cell (108, 109). Leukotrienes appear to have similar effects in the newborn. Rooney and Gobran (252) have demonstrated that the increase in surfactant secretion in the newborn rabbit is blocked by LT receptor antagonists, as well as by a lipoxygenase inhibitor, suggesting that LTs play a physiological role in the regulation of lung surfactant secretion and the changes that occur around birth.

Hyperoxia

High levels of oxygen administered during alveolarization have been shown to alter the development of alveoli in many animal models (Table 4). Hyperoxia is also a major pathogenic factor of bronchopulmonary dysplasia (BPD), which is a chronic lung disease of prematurity. Babies with BPD exhibit many of the same changes in lung structure and function including inhibition of septation and airway hyperreactivity(1, 211), as seen in animals exposed to hyperoxia. Hyperoxia's effects on the lung and the mechanisms involved are the subjects of the following section.

C. THE EFFECT OF HYPEROXIA ON THE DEVELOPING LUNG

i) Pathology of Hyperoxia-Induced Lung Damage

The pathological changes caused by normobaric hyperoxia are primarily limited to the lung, except for the unique toxicity that O_2 may cause to the eyes of the newborn. The effects of hyperoxia on the lung have been well characterized in a variety of species (for examples see Table 4). These studies have shown that the first visible signs of lung injury after hyperoxic exposure are a swelling of endothelial cells and an altered appearance of mitochondria and microsomes (66). Later, there are shifts of lung cell populations as a result of proliferation and hypertrophy of type II cells, and loss of capillary endothelial cells. There is a major increase in intrapulmonary neutrophils and an inflammatory response occurs (64). Often, both pulmonary endothelium and epithelium are damaged, which results in alveolar proteinosis (41, 103).

What is evident is that the sequence of injury moves outward from the endothelium to the epithelium, even though it is the epithelium that is directly exposed to oxygen radicals. There are two broad patterns of damage. Both type I cells and endothelial cells are damaged and possibly destroyed by hyperoxia, with the endothelial cells succumbing early and type I cells showing initial resistance and later injury. Type II cells actually proliferate at the same time as other cell types are being destroyed.

When newborns are exposed to hyperoxia, it has a profound impact on postnatal lung growth. Because alveolarization occurs postnatally, it has the potential to be altered by extreme changes in the amount of oxygen it is exposed to. Septation is quite noticeably inhibited by hyperoxia, resulting in fewer and larger alveoli. Secondary septation can be inhibited by as much as 88% and whole lung DNA reduced to 50% of control (41). This problem can grow bigger with the development of interstitial and alveolar fibrosis. So, by the end of hyperoxic exposure, septa are fewer and thicker making the lungs less compliant (30). These effects of hyperoxia on the growing lung are especially unique to newborns, since they are not observed during compensatory lung growth in the adult lung (260). Another effect of hyperoxia in the newborn is the development of airway hyperresponsiveness which does not occur in the adult (135, 212).

ii) Mechanisms of Oxygen Toxicity

Free Radicals

The "free radical theory of oxygen toxicity" attributes the damaging effects of hyperoxia to highly reactive metabolic products of oxygen that can inactivate enzymes in the cell, damage DNA, and destroy lipid membranes (Figure 2). To protect the organism from this insult, cooperative antioxidant defense mechanisms are involved. The increased levels of radicals released during hyperoxia may overwhelm these systems. Having reduced antioxidant capability during normoxic conditions may also have detrimental effects.

Oxygen free radicals are products of normal cellular functions (oxidation-reduction processes). Hyperoxia increases their numbers dramatically. The oxygen molecule can form the superoxide anion [O^-_2], hydrogen peroxide [H_2O_2], the hydroxyl radical [$OH\cdot$], and singlet oxygen [$^1O_2$]. These radicals can inactivate sulfhydryl enzymes, destroy DNA and cause lipid peroxidation of cellular membranes (124, 159, 174).

Since pulmonary O_2 toxicity is thought to be mediated by oxygen (102, 104), much attention has been focussed on antioxidant enzymes. Antioxidants could play a crucial role in the response to oxidant stress by scavenging the radicals (103, 201, 229). In adults, antioxidant enzymes respond differently to hyperoxia in different species, and there is no simple "dose response" to hyperoxia. Rats exposed to 80-85% O_2 exhibit modest increases in the activity of the classical antioxidants superoxide dismutase (SOD) (65, 298) and glutathione peroxidase (GP) (157), whereas exposure to 100% O_2 results in decreased catalase (CAT) in adult rats (6). In adult rabbits, SOD, CAT, and GP enzyme activities do not increase at all in hyperoxia (14). Newborn rats exposed to hyperoxia show increased lung CAT (58). The activities of SOD, CAT, GP are induced by hyperoxia in newborn (term) rabbits, but not in premature rabbits (101). Isolated type II cells from newborn rabbits exposed to 100% O_2 secrete increased extracellular GP (145). This may explain why the neonates of some species are relatively more resistant to hyperoxia than adults (95, 312).

The question as to whether endogenous increase in any of these enzymes in response to hyperoxia offers protection is unknown. Transgenic mice that overexpress Cu,Zn-SOD or GP are not more resistant to 100% O_2 than their nontransgenic counterparts (221, 306). Also, prematurely weaned newborn rats are more tolerant of hyperoxia than normally weaned littermates, yet their antioxidant enzyme activity levels are indistinguishable (99).

Mediators of Oxygen Toxicity

While there is no doubt that oxygen radicals cause damage, there is a series of events initiated by this primary insult that results in the formation and release of various chemical mediators. One must then ask how much of the damage is a result of the direct damage due to radicals and how much is due to that of these mediators? In several animal models, it has been shown that hyperoxia stimulates various genes leading to the formation of numerous compounds which may have an effect on the developing lung.

Tables 2 and 3 show several substances whose levels are increased or decreased due to hyperoxic exposure. Some of the ones that are particularly interesting are briefly discussed below.

Growth Factors and Cytokines

Many aspects of the pathophysiology of hyperoxic lung injury seem likely to be mediated by growth factors and cytokines. For example, a number of cytokines have a role in the initiation and propagation of inflammation, a hallmark of pulmonary O_2 toxicity. In addition, cell hyperplasia that occurs during tissue remodeling from hyperoxia may also involve growth factors. In hyperoxic rats, c-sis mRNA, which encodes the B-chain of platelet derived growth factor (PDGF), increases two- to threefold after 3 days of exposure to 85% O_2, and this persists up to 7 days (123). Prolonged sub-lethal hyperoxia induces the appearance of new contractile cells in the pulmonary vessels, which proliferate at between 4 and 7 days of hyperoxia. The level of mRNA encoding epidermal growth factor (EGF) increased 100 fold by day 7 of hyperoxia (242). The cytokine interleukin 1-β (IL-1β) is also increased in adult mice exposed to 100% O_2 and may be important (145).

Leukotrienes

Leukotriene levels are increased in various animal models during hyperoxic exposure (236, 270, 276). Stenmark *et al.*(279)measured an increase in the levels of lipid mediators in BAL fluid of infants with bronchopulmonary dysplasia (BPD). Lipoxygenase products and TXB_2 were elevated to a greater extent than was 6-keto-

$PGF_{1\alpha}$. Increased levels of LTB_4 in BAL of infants with BPD shows correlation with changes in airway reactivity (211).

Leukotriene involvement in newborn hyperoxic lung injury has not been widely studied, despite the fact that investigations like those mentioned above suggest that they may have a role. The actions of LTs are quite similar to many of the things seen in hyperoxic lung injury. Leukotrienes and their actions are the subject of the next section.

D. LEUKOTRIENES

i) Leukotriene Actions

Leukotriene B_4

Leukotriene B_4 (LTB_4) secretion by myeloid cells as well as by nonmyeloid cells caused by transcellular metabolism induces a range of cellular and molecular responses that coordinate and amplify the inflammatory response. The chemotactic, chemokinetic, and vasoactive properties of LTB_4 are the best known, but this molecule may also possess other activities from mediation of pain to modulation of diverse immune responses.

LTB_4 is probably the most potent neutrophil chemotactic agent produced by the AA cascade (111, 226). Intratracheal installation of LTB_4 induces the selective recruitment of functionally active neutrophils into human BAL fluid (194).

LTB_4 may play a pivotal role in the induction of neutrophil-endothelial cell adherence (143, 288). *In vivo* the topical application of LTB_4 to the vascular network within the hamster cheek pouch results in immediate and reversible adherence of neutrophils to venular endothelial cells (72). LTB_4-induced endothelial cell hyperadhesiveness for neutrophils depends on increased CD11/CD18 expression on the neutrophil surface and possibly a specific domain of the adhesion molecule CD54 found on endothelial cells (225, 289).

At nanomolar concentrations, LTB_4 causes the release of substantial quantities of glucuronidase and lysozyme from neutrophils, although less effectively than the chemotactic fragment of the complement component C5 (20, 262). LTB_4-induced enzyme secretion is mediated by LTB_4 recognition of a surface receptor of substantially lower affinity than that which mediates neutrophil aggregation, adherence to endothelial cells and chemotaxis.

In vitro, LTB_4 stimulates myelopoiesis (54, 279). Activation of nuclear factors NFκB and NF-IL-6 transcriptional factors may be important in this enhancement of IL-6 release (37). LTB_4 may modulate the production of other cytokines by stimulating gene transcription of proto-oncogenes c-fos and c-jun in mononuclear cells (277).

Peptido-Leukotrienes

The biological activity of peptido-leukotrienes (peptido-LTs) differs substantially from that of LTB_4. They were first identified as the constituents of the slow-reacting substance of anaphylaxis (213). Their capacity for inducing airway, gastrointestinal, and mesangial smooth muscle contraction is probably the best characterized of the biological activity of these molecules (131, 258). Like LTB_4, the peptido-LTs play numerous roles in the inflammatory process, inducing vasoconstriction, increasing vasopermeability, enhancing mucous secretion, and acting as immunomodulatory agents (131, 258).

Smooth muscle contraction

In general, the peptido-LTs have potency as smooth muscle spasmogens approximately 1000 times that of histamine (73, 90). They cause sustained muscle contractions when applied to guinea pig tracheal and lung parenchymal preparations *in vitro* at picomolar doses (129). The contractions caused by LTs are of a significantly more sustained nature than those that occur after treatment with histamine.

Modulation of Vascular Permeability and Vasoconstriction

The increase in the permeability of the venular endothelium that allows proinflammatory cells to migrate to the site of inflammation is thought to be driven by the peptido-LTs (154, 232). Plasma components also leak into the extra-vascular tissue, leading to edema (72, 273).

The smooth muscle contractile effects of peptido-LTs both in lung strips and during inhalation affects the vascular smooth muscle. Leukotriene C_4 and LTD_4 induce vasoconstriction of guinea pig skin (90) and the cheek pouches of hamsters (72). LTC_4 causes powerful vasoconstriction of rat mucosal vessels leading to stasis of blood between constricted areas (307). *In vivo* infusion of LTC_4 and LTD_4 into a major coronary vessel of sheep and systemic venous circulation of rat produces coronary vasoconstriction; the systemic response to LTC_4 in the rat includes renal vasoconstriction (12, 205).

Mucous secretion and Immune Modulation

Peptido-LTs enhance mucous secretion in the airways. LTC_4 and LTD_4 are strong inducers of mucous secretion in human bronchial explants *in vitro* (62, 192) and in the trachea of dogs (152) and cats (231) *in vivo*.

Leukotriene C_4 and LTD_4 stimulate expansion of myeloid colonies treated with colony-stimulating factors (207). They may also be involved in the proliferative development of glomerular epithelial cells (22) and fibroblasts (21) *in vitro*. LTD_4 enhances IL-4 production by human monocytes and can replace IL-2 in the induction of IFN-γ secretion by T-lymphocytes.

ii) Leukotrienes in Lung Pathology

Over the past 30 years, the concept that the *in vivo* modulation of levels of AA and oxygenated metabolites of AA (including the LTs) is intimately linked to inflammation and disease has firmly been established, at least in terms of diseases like asthma. While a low level of specific eicosanoids may render a protective response enhancing disease resistance, these molecules may induce an autotoxic response when produced in excessive quantities. It is not clear what initiates "crossing the line".

LTs are released in several inflammatory lung conditions. They are found in the sputum of patients suffering from lung diseases including cystic fibrosis (67), asthma (316), and chronic bronchitis (315). Fragments of lung and peripheral blood leukocytes from patients with allergic asthma release LTs when challenged with the appropriate antigen *in vitro* (73). Further, LTs are released in higher quantities from stimulated leukocytes after an asthmatic attack (11). They are found in the BAL fluid of patients with several forms of asthma including bronchial asthma where LTs including LTE_4 and the metabolite of LTB_4, 20-OH LTB_4, have been found (170). Resident lung cells such as mast cells and macrophages could mediate initial events (142). Cells, especially eosinophils, could play an important role in LT release. As mentioned in a previous section, LT levels are also increased in infants with the lung disease BPD.

iii) Leukotriene Inhibitors and Antagonists

Several approaches have been adopted to inhibit or suppress leukotriene activity (97, 214, 257). Inhibiting 5-LO leads to suppression of the formation of both LTB_4 and peptido-LTs. The 5-LO inhibitor most studied in clinical trials is zileuton. Zileuton inhibits the active-site iron of 5-LO at concentrations that do not inhibit cyclooxygenase, 12-LO, or 15-LO (51). Leukotriene formation can also be inhibited by compounds that bind to FLAP. This prevents the translocation of 5-LO to the cell membrane. MK-0591 is a representative FLAP inhibitor that blocks LT synthesis and is effective in clinical situations (82, 86, 281). An alternative approach is to block the actions of LTB_4 and the peptido-LTs by specific receptor antagonists. The availability of LT inhibitors and antagonists have helped elucidate the role of LTs in many inflammatory diseases.

Initial studies using novel LT receptor antagonists and inhibitors have confirmed a prominent role for LTs as mediators of diseases such as asthma. In exercise-induced asthma, pretreatment with the selective LTD_4 antagonist MK-0571 attenuates the bronchospastic response to exercise challenge (190). MK-0571 was reported to be a potent antagonist of LTD_4-induced bronchoconstriction in both normal volunteers and asthmatic patients. It also caused a small, but significant increase in baseline airway caliber in asthmatic patients, suggesting the presence of LTD_4 in asthmatic airways and providing further evidence supporting a role for LTs in the pathogenesis of airway diseases such as asthma (162). Another LTD_4 receptor antagonist, ICI 204,219, has been shown to inhibit both early- and late-phase asthma responses after inhalation of specific allergen (283). In guinea pigs, the selective LTB_4 receptor antagonist U-75302 inhibits both LTB_4-induced lung eosinophilia *in vitro* and antigen-induced lung eosinophilia *in vivo* (248). Other receptor antagonists such as MK-679 also have effects.

The FLAP inhibitor MK-886 partially inhibits early- and late-phase asthma responses after allergen challenge (105). Specific inhibition of 5-LO by zileuton significantly improves the bronchospastic response to inhalation of cold, dry air in asthmatics (150).

The use of LT inhibitors is a very useful tool in evaluating the effects of LTs. In this study, WY-50295, a 5-LO inhibitor and LTD_4 receptor antagonist, MK-0591, a FLAP inhibitor, and MK-0571, an LTD_4 receptor antagonist, were used to investigate the role of LTs in hyperoxia-induced lung injury. Wy-50,295 is a potent and orally active 5-LO inhibitor in a number of *in vitro* and *in vivo* systems, and has a long duration of action in an allergic bronchoconstriction model (118, 128). However, it has been found to have some anti-PAF activity (140). MK-0591 is an extremely potent LT synthesis inhibitor through its ability to bind with high affinity to FLAP (82, 86, 281). MK-0571 is quite potent in man (83).

E. MODELS FOR STUDYING THE EFFECTS OF HYPEROXIA ON THE LUNG

Wessler defined an animal model as "a living organism with an inherited naturally acquired, or induced pathologic process that in one or more respects closely resembles the same phenomenon in man (305)." The ideal model would be one in which the disease occurred spontaneously with sufficient frequency in a species with developmental, metabolic, physiologic, and anatomic similarities to humans.

Hyperoxic lung injury rarely occurs on its own in newborns and is more likely to be one of a number of factors that contribute to the pathogenesis of BPD. The degree of prematurity of the lung is another factor involved in this disease, as are barotrauma, and infection. At this time there is no readily reproducible, naturally occurring model of BPD (63). However, we are able to study this disease by teasing apart the various factors that may be involved, including hyperoxia.

Various models studying the effects of hyperoxic exposure have been developed (Table 4). The best models in terms of the effects on the developing lung will of course be those models involving newborn animals whose lungs are immature and which have pathogenesis similar to that seen in the human. Good models will also be easily manipulated. Although unfortunate, monetary considerations must also be kept in mind. Thus, there are few models that actually take all of these things into consideration.

When one thinks of a model for human disease, naturally one thinks of primates. Using a primate model would involve delivering newborns prematurely before alveolarization starts or very close to when it begins and then maintaining these babies on ventilators, etc. The disadvantage of this would be the numerous interventions that would have to be made to keep these animals alive for a long enough time to study them. While, the lungs would be premature, so would numerous other organ systems, confounding the investigation of the lung. Setting up such facilities is very expensive and the number of animals that could be studied is quite small compared to some other studies.

The rat pup makes a very good model for studying the effect of hyperoxia on alveolarization for a few reasons. First, rapid alveolarization occurs postnatally in a relatively discrete time period (days 4-14). This makes the need for a premature model and all the complications that it involves, unnecessary. The administration of hyperoxia is easier in that a whole litter of pups and their dams can be placed in a chamber and exposed for various periods of time. Their size is quite manageable, and they are relatively inexpensive.

There is one disadvantage to the rat pup model. The rat pup is obviously able to survive quite happily with its lungs born into the saccular stage, unlike human babies, who require much intervention on their behalf. The rat pup, while immature structurally, is quite well prepared biochemically for life in the extrauterine world (in terms of antioxidant enzymes and surfactant for example). To properly study the effects of hyperoxia on the developing lung, the biochemistry of the lung as well as the structure should be immature. However, tipping the balance in terms of the oxidants over the antioxidants means increasing oxygen levels to extremely high levels, not usually experienced by most premature babies for such a long period of time. Despite this, the rat pup remains an excellent model for studying lung development.

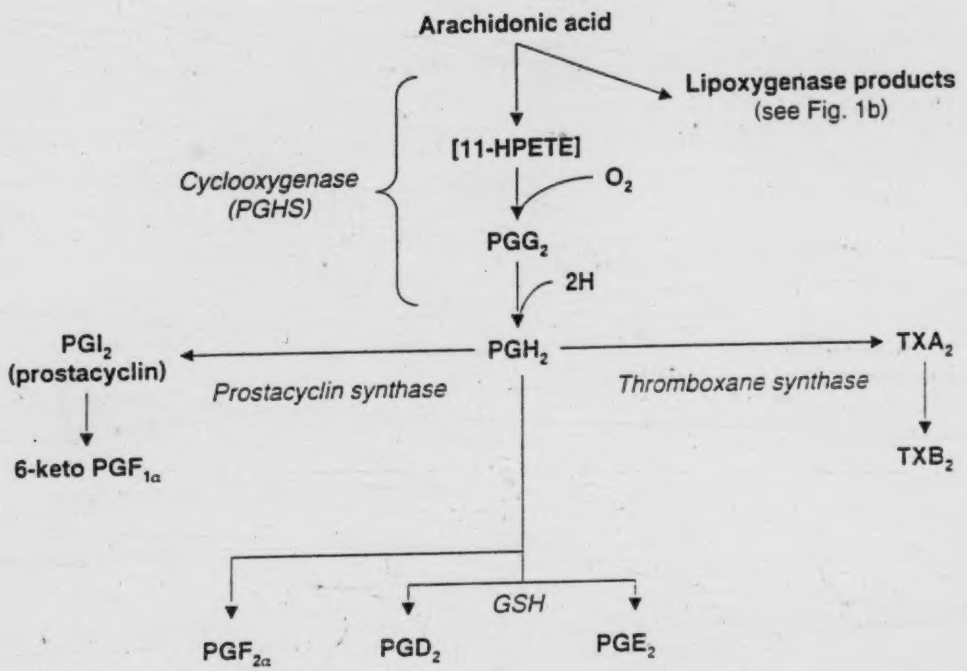


Figure 1a. Principal products of cyclooxygenase (prostaglandins and thromboxanes). *Modified from Cunningham, F.M. (1994)* *The Handbook of Immunopharmacology,* *pp.375.*

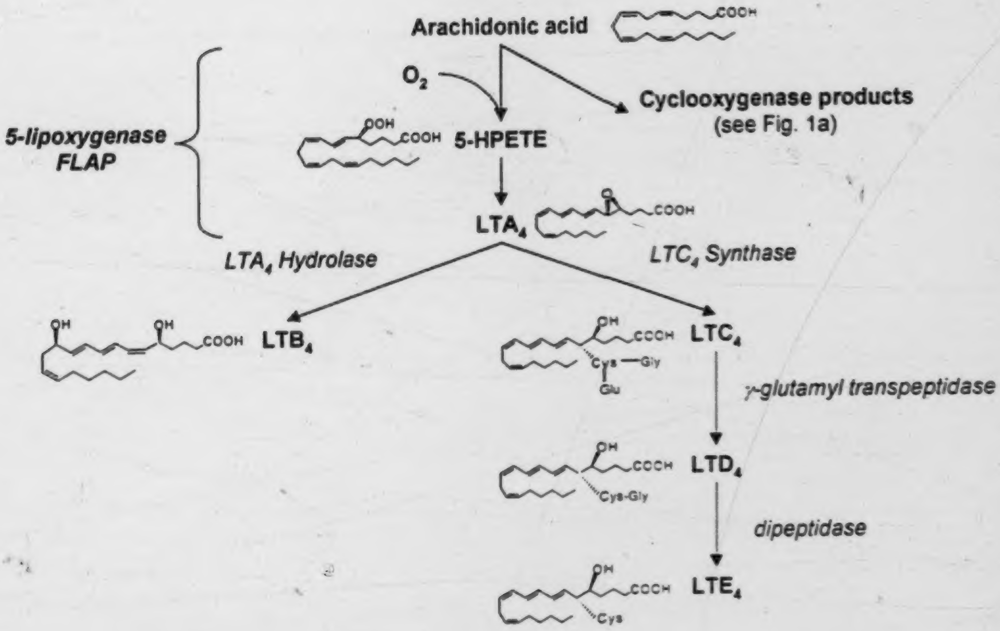


Figure 1b. Principal 5-lipoxygenase products. *Modified from Cunningham, F.M. (1994) The Handbook of Immunopharmacology, pp.375.*

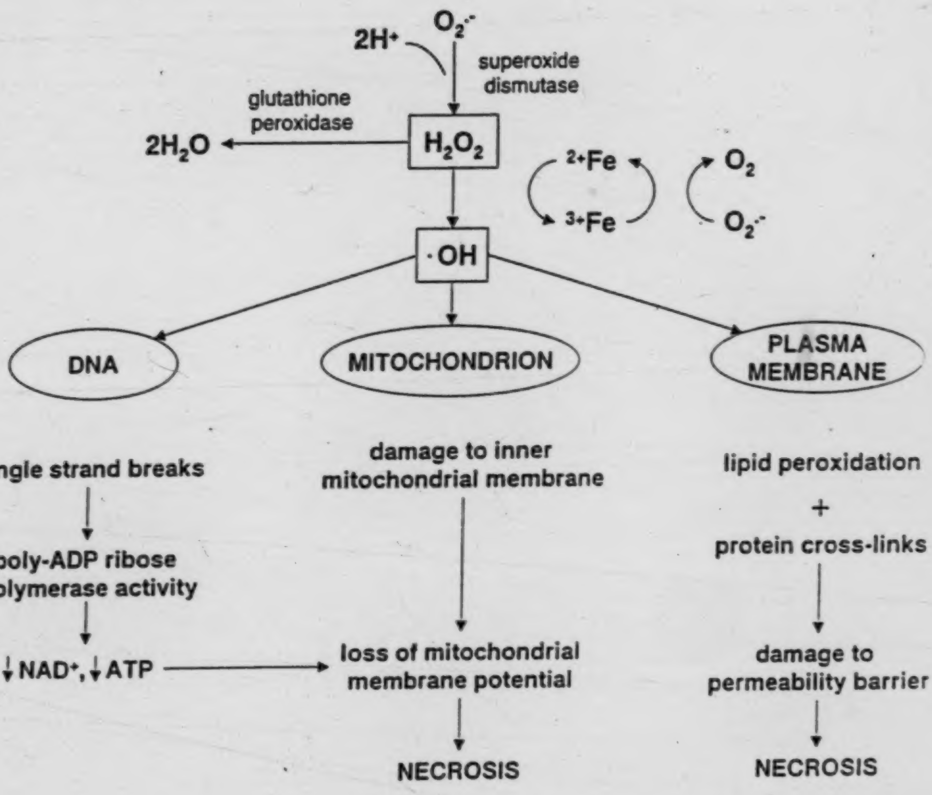


Figure 2. Proposed mechanism of hyperoxic or oxidant-induced injury. Iron-catalyzed generation of the hydroxyl radical is the key initiating event. This highly reactive radical targets all intracellular components. Lipids in the plasma membrane and endoplasmic reticulum are damaged by a self-propagating chain reaction (lipid peroxidation) and proteins are cross-linked at sulfhydryl groups destroying key enzymes. Mitochondria are especially sensitive to hyperoxia as is the genome of the cell. The hydroxyl radical causes oxidized bases and DNA strand breaks, which may actually lead to apoptosis in some cases. *Modified from Kane, A.B. (1996) Mechanisms of Cell and Tissue Injury in Cellular and Molecular Pathogenesis, Sirica, A.E. ed., Lippincott-Raven, Philadelphia, pp.557.*

Table 1: Growth Factors and Cytokines in Alveolar Development

Animal/Strain*	Characteristics	References
BMP-4 +	large cyst -like alveoli	(25)
Calmodulin inhibitor +	branching inhibited, lungs lack epithelium	(302)
C/EBP-α -/-	hyperproliferation and immaturity of alveolar type II cells	(96)
EGF-R -/-	thicked-walled alveoli, extended terminal bronchi	(206)
EGF-R -/-	undifferentiated epithelium in respiratory bronchi and alveoli	(267)
FGFR2 +	lungs are 2 bifurcated tubes, no branching	(234)
Glucocorticoid receptor -/-	lung atalectasia, reduced surfactant	(27)
GM-CSF -/-	develops alveolar proteinosis, progressive accumulation of surfactant lipids and proteins	(89)
IGF-I -/-	generalized organ hypoplasia and growth defects	(13, 182)
IGF-II -/-	small but otherwise normal	(78)
IGF-I R -/-	more severe developmental delays than IGF-I null mouse, die at birth of respiratory insufficiency	(13, 182)
IGF-II R -/-	retarded lung development,alveoli poorly formed	(304)
Integrin α-3 +	no bronchioles, bronchi extend to periphery, alveoli form	(169)
KGF (FGF-7) +	lungs cystic with dilated saccules	(303)
KGF (FGF-7) +	causes proliferation of respiratory type II epithelium	(219)
Megalin/gp330 -/-	impaired pulmonary inflation and alveolar expansion	(309)
PDGF-A -/-	no alveolar septation	(35)
TGF-α +	disrupted alveolar development	(166)
TGF-β1 +	morphogenesis arrested at late pseudoglandular stage	(320)
TGF-β3 -/-	cystic alveoli	(156)
VEGF +	premature sacculation	(319)

* -/- indicates a knockout strain for that particular substance. + indicates overexpression of the substance.

Table 2: Some Substances in the Lung Increased by Exposure to Hyperoxia

MODEL	SUBSTANCE	References
Rat	SOD, Heme oxygenase-1, c-fos, c-jun, CAAT-enchancer bP	(53)
Adult Mouse (100% O_2, 72 hrs)	ICAM-1	(237)
Human type II pneumocytes (cultured, 90% O_2)	ICAM-1	(280)
Pulmonary microvascular endothelial cells (hyperoxia, 24 hrs)	HOX-1	(299)
Newborn Rat (hyperoxia, 3 days)	HOX-1 activity (postranscriptional)	(79)
Mouse (<95% O_2)	CD54 (ICAM-1) mRNA CD11a, CD62 TNFα mRNA	(17)
Rat (bronchiolar epithelium, interstial and inflammatory cells)	HOX-1 mRNA HOX-1 protein, enzyme activity	(176)
cultured epithelial cells, fibroblasts, macrophages, smooth muscle cells	HOX-1 gene, mRNA, protein AP-1 activation	(178)
Chinese Hamster fibroblasts	HOX-1	(80)
Rat (21 d, 100% O_2, 8d) fibroblasts	sensitivity to IGF-1 c-Ha-ras	(158)
Neonatal Rabbit (>95% O_2, 8d)	SP-B mRNA (type II cells) SP-C mRNA	(75)
BAL cells from 1-24h or 3-28 hrs)	TNF-α	(144)
Adult Rat (>85% O_2)	Mn-SOD mRNA	(139)
Rat type II cells (>90%O_2, 48 hrs)	EGF EGF receptor	(220)
Rat (100% O_2)	TNF	(289)
Newborn Rat	collagenase type I and IV	(85)
Adult Rat (95% O_2, 60hrs)	ferritin mRNA	(255)
Neonatal Rat (7d)	Hyaluronan CSPG (chondroitin sulfate proteoglycan)	(155)
Adult Mouse	Plasminogen activator inhibitor-1 (PAI-1)	(16)
Adult Mouse	NF-κB (lymphocytes), TNF-α, IFN-γ	(265)
Human Type II Cells (80% O_2)	Vitamin E	(287)
Neonatal Rabbit, alveolar epithelial cell (100% O_2, 9d)	VEGF (during recovery phase)	(189)
Newborn Rat, (>95% O_2, from d1-7)	6-keto $PGF_{1\alpha}$, TxB_2, LTB_4	(167)
Newborn Rat (>95% O_2,from d1-28)	TXB_2, LTB_4	(167)

Table 3: Some Substances in the Lung **Decreased** by Exposure to Hyperoxia

MODEL	SUBSTANCE	References
Neonatal Rabbit alveolar epithelial cell (100% O_2, 9 d)	VEGF TGF-β (during recovery phase)	(189)
Rat	PGE_1 metabolism PGDH activity	(238)
Newborn Rat (>95% O_2, 1d)	6-keto$PGF_{1\alpha}$, TxB_2	(167)

Table 4: Models used in studying hyperoxic lung damage

Animals	O_2 Exposure	Effect	References
Newborn mice	100%, 8 days	no lung pathology	(239)
Adult mice	100%, 8 days	died, exudative lung changes	(239)
Newborn mice	100%, 7 days	ultrastructural lung lesions	(34)
Newborn mice	100%, 7 days	necrotizing bronchiolitis	(187)
Newborn mice	100%, 5-25 days	lack of exudative response, mixed emphysema, patchy atelectasis, connective tissue proliferation, hemorrhages	(130)
Newborn mice	100%, 6 weeks	progressive pulmonary changes, gross emphysema at 3 weeks, airway lesions and alveolar septal fibrosis at 46 weeks	(34)
Newborn mice	80%, 6 weeks	increase in peribronchiolar and parenchymal fibrosis compared to 100% O_2, decreased cellular response of type II and bronchiolar cells	(34)
Newborn rats	various levels, 6 days	# of capillaries/mm^2 decreased, increased capillaries compared to controls after recovery for 2 weeks	(251)
Rats (1 day old)	45.8%, 15 days	decreased lung weight and volume, alveolar surface area, alveolar #	(19)
Rats (4 weeks)	100%, 4 weeks	progressive lung damage, bronchiolar hyperplasia/metaplasia at 4 weeks, emphysema at 5 weeks	(40)
Adult Rats	85%, 7-10 days 100%, 6 weeks	if survived 7-10 days 100% O_2, extensive fibrosis, emphysema	(286)
Adult dogs	60%, 33 days	bullous emphysema, 2 to 3x increase in right ventricular size	(40)
Newborn rats	95%, 7 days	enlarged airspaces at 40 days	(245)
Premature baboons	100% 6-10	diffuse alveolar damage, alveolarization normal at d21	(60)
Newborn rats	96-100%, 8 days	decreased density of arterioles, abnormally thick walled arteries, elevation in right ventricular pressures, right ventricular hypertrophy several weeks after	(263)

II. RATIONALE

A thorough understanding of the process of alveolarization in the lung is essential due to its implications for disease prevention and treatment in preterm newborns. In normal lung development of rats, where alveolarization occurs postnatally, or in humans, in whom the majority of lung alveolarization occurs postnatally, the physiological demands of the newborn may regulate the process. At present, little is known about the mechanism whereby metabolic need is translated into morphological outcome.

Challenging the process of alveolarization by prematurity or adverse levels of oxygen, leads to definite immediate changes in lung morphology and health, the consequences of which can last well into adulthood. Because they are capable of eliciting actions which may be important during the pathogenesis of hyperoxic lung damage (edema formation, smooth muscle contraction, chemoattractant, etc.), the leukotrienes are one group of mediators that may be involved in hyperoxia-induced lung injury. Leukotriene levels have been shown to be raised in the BAL fluid of infants with BPD and in various animals exposed to hyperoxia.

The objective of this study, therefore, is to establish a link between lung damage induced by exposure to hyperoxia during the alveolar stage of lung development and LTs. The specific aims are:

1. To examine whether inhibition of LT synthesis in rat pups exposed to hyperoxia during the period of alveolarization (postnatal days 4-14) prevents inhibition of septation measured at 14 days of age.

2. To examine whether inhibition of LT synthesis in rat pups exposed to hyperoxia from days 4-32 prevents the changes in airway smooth muscle or hyperreactivity to methacholine measured at 5 weeks of age.

3. To determine whether there is a "critical period" during alveolarization when hyperoxia has a more profound effect and whether this period is associated with a critical period for LT effects.

4. To describe the location of 5-LO in the lungs of hyperoxic and normoxic rats at day 14.

5. To examine whether LTD_4 receptor antagonism in rat pups exposed to hyperoxia during the period of alveolarization (postnatal days 4-14) prevents inhibition of septation measured at 14 days of age.

6. To determine whether administration of LTC_4 or LTD_4 to normal rats during the period of alveolarization (postnatal days 4-14) alters postnatal lung development measured at 14 days of age.

III. MATERIALS AND METHODS

A. ANIMALS

Sprague-Dawley albino rat pups (Charles Rivers Laboratories, St. Constance, QC, Canada) of both sexes were used in all experiments. They were housed in the Health Sciences Laboratory Animal Service of the University of Alberta under veterinary care. The guidelines of the Canadian Council of Animal Care were followed in all experimental procedures. Dams were maintained on regular laboratory rodent pellets and water *ad libitum* and kept on a 12:12 hour light-dark cycle. Pups were weaned from the dams on day 21.

B. OXYGEN EXPOSURE AND LT INHIBITION

Parallel litters of randomly divided rat pups and their dams were placed into 0.14m^3 Plexiglas chambers and maintained in hyperoxic or normoxic conditions. Oxygen concentrations were monitored daily (Ventronic oxygen analyzer no. 5517, Temecula, CA, USA). Oxygen and air were filtered through barium hydroxide lime (Baralyme; Chemtron Medical Division, St. Louis, MO, USA), to keep CO_2 levels under 0.5%, and through activated charcoal. Temperature and humidity were maintained at 26°C and 75-80% respectively. Chambers were opened for 15 minutes daily to switch dams between cages and administer drugs if required.

Alveolarization Study

Rat pups were maintained in >95% O_2 from days 4-14 of life. On day 14 pups were removed from the oxygen and various procedures performed. Leukotriene inhibition was achieved using the drugs WY-50295 (5-LO inhibitor and LTD_4 receptor antagonist, 30 mg/kg; a gift from Wyeth Ayerst, Princeton, NJ, USA), MK-0591 (FLAP inhibitor, 20 mg/kg; a gift from Merck-Frosst, Ponte-Claire, QC, Canada), or MK-0571 (LTD_4 receptor antagonist; a gift from Merck-Frosst, Pointe-Claire, QC, Canada), which were administered subcutaneously (sc) or orally (po) from days 3-14. Each drug group had its own control vehicle group.

Pulmonary Function Study

Pups were maintained in >95% O_2 from days 4-14, when they were lowered to 65%. Wy-50295 (30 mg/kg) was adminstered sc to rat pups from days 3-14 and orally from day 15 onward. The vehicle consisted of a mixture of 1 unit of Tween 80 with 4 units of distilled H_20 for sc adminsitration or 1 unit of Tween 80 with 4 units of 1.5% methylcellulose for po adminstration.

Critical Period Study

Pups were maintained in >95% O_2 from days 1-4, 4-9, or 9-14 after birth. In another experiment, pups were exposed to >95% O_2 from days 4-14 and MK-0591 was administered from days 4-9, 9-14 or 4-14.

C. PEPTIDO-LT PRODUCTION

Lung and airway peptido-LT production was measured using a short-duration lung explant technique. Briefly, pups were killed with an overdose of pentobarbital (100 mg/kg Euthanyl; MTC Pharmaceuticals, Cambridge, ON, Canada). The distal trachea with the extra-pulmonary part of the main bronchi or three 500 µm thick blood-free lung slices (sliced using a tissue slicer from Stoelting Co. (Wood Dale, IL, USA)) were placed in tissue culture wells (12-well plate; Costar, Cambridge, MA, USA) containing 800 µl of culture medium (Hanks buffered salt solution with HEPES to pH 7.36, 1.67 mM $CaCl_2$) and were incubated at 37°C for 30 min. Culture medium was stored at -70°C before being assayed for LT levels using enzyme-linked immunosorbant assay (ELISA).

Peptido-LTs were assayed using a LTC_4/LTD_4/LTE_4 ELISA kit From Oxford Biomedical Research or Cayman Chemicals. Typical results for the Oxford kit yield 50% of the sample or standard bound/medium bound (B/Bo) at 1.93ng/ml and and 80% B/Bo at 0.83 ng/ml. The specificity of the kits used was 100% for LTC_4, >80% for LTD_4 and LTE_4, <2% for LTA_4 and <1% for LTB_4. The 6-keto-prostaglandin$F_{1\alpha}$ (6-keto$PGF_{1\alpha}$) production was quantified by radioimmunoassay. 6-keto[5,8,9,11,12,14,15(n)-3H]$PGF_{1\alpha}$ was obtained from Amersham. The 6-keto $PGF_{1\alpha}$ antibody (Cayman) was used at a 1:15,000 dilution. The total activity of the 6-keto$PGF_{1\alpha}$ was 7,000 -10,000 cpm and the concentrations of standard used were 3.8-1,000 pg/ml. All eicosanoid levels were normalized by the total DNA (assay modified from Downs and Wilfinger (88)) contained in the lung tissue. Briefly, 1.5 ml DNA extraction solution (1M NH_4OH, 10mM EDTA) was added to the tissues in plastic tubes, which were then homogenized at grade 5 for 20 seconds (lung slices) or 30 seconds (trachea). The tubes were incubated at 37°C for 30 minutes and 500µl of the homogenate was centrifuged at 10,000xg for 3 minutes. 25 µl of supernatant was transferred to a test tube and 50µl of H_2O was added. This solution was dried in an evaporating centrifuge for 11 minutes, until the pH was ~7.5. Enough H_2O was added to bring the volume up to 500µl.

1.5ml of the dye solution (50µl Hoeckts 33258 (200µg/ml) + 100ml DNA assay buffer (100mM NaCl, 10mM EDTA, 10 mM TRIS, pH=7.0)) was added to 50µl of the sample or standard (DNA from salmon testes, Sigma Chemical Co.), vortexed and left at room temperature for 30 minutes. Measurements were made with a F-2000 fluorescence spectrophotometer (Hitachi) at a wavelength of 350nm.

D. MEASUREMENT OF ACUTE LUNG DAMAGE

Extravascular lung water was assessed in rats by calculating wet-to-dry weight ratios. Animals were killed and exsanguinated. Then lungs were removed from the chest cavity and blotted minimally, and the wet weights were determined. The lungs were then allowed to dry in a vacuum oven for 2 days at 65°C. The tissue was considered dry when the dry weight was constant for two consequtive days.

Total protein in bronchoalveolar lavage fluid (BALF) was also measured. Physiological saline (1 ml) was instilled into the trachea via a polyethylene cannula [inner diameter 0.86 mm, outer diameter 1.27 mm, Intramedic, Becton-Dickson, Parsippany, NJ, USA] and then recovered. This was repeated two additonal times, and all recovered fluid was combined. Samples were only used if >80% instilled saline was recovered. The BALF was then centrifuged for 5 minutes at 200xg. Total protein in the supernatant was measured using a Bradford (Bio-Rad Laboratories, Hercules, CA, USA) microassay using bovine serum albumin (Sigma Chemical, St. Louis, MO, USA) as the standard.

E. LUNG MORPHOMETRY

Lung Preparation

Lungs were fixed *in situ* through a polyethylene tracheal cannula with 2.5% glutaraldehyde at a constant pressure of 20 cm H_2O for 2 hours. Then the trachea was ligated, and the lungs were excised and immersed in glutaraldehyde for 24 hours. Lung volumes were measured by water displacement before and after the 24 hours fixation period to detect shrinkage. Because of minimal shrinkage (0-2%), data did not have to be corrected.

After fixation, transverse sections of superior, middle, and inferior portions of right lung and superior and inferior portions of left lung were imbedded in paraffin. The entire transverse sections were cut 3 μm thick and stained with Gomori-trichromaldehyde fuschin. Slides were initially examined to eliminate sections with evidence of inadequate preparation.

Parenchymal Morphometry

Light level morphometric assessment of lung parenchymal tissue was performed in a blinded fashion on coded slides from 6-15 animals from each experimental group. Ten randomly selected fields were examined from each lung. Histological specimens observed with the microscope (Carl Zeiss Jenamed Variant) were put into a gray image analyser system (IS Tech) via a video camera (MTI S 68). The measurements and calculations were performed using Genias 25 Image Analysis software (Joyce-Loebl).

Parenchymal tissue includes alveolar septa, alveolar ducts, respiratory bronchiolar tissues, and blood vessels with a diameter ≤ 10 μm and their contents. Volume density of perenchymal tissue (V_p) was calculated as [field area (FA)-airspace area (A_{asp})/FA X 100 from each analyzed field. Mean septal thickness (Th_{sept}) was calculated from parenchymal tissue area and length of gas exchange surface.

As an indication of mean alveolar diameter, the mean linear chord length (Lm) was calculated by dividing the length of the computer-generated horizontal test line by the number of intercepts of the septal wall. Mean volume of airspace units ($V_{aspunits}$) was computed as (Lm^3 x p)/3. To detect the structural changes of alveolar airspaces (i.e. the shape), the perimeter (of airspace)-to-area (of airspace) ratio (P/A) of the airspace was calculated from each field. Perimeter/area gives an indication of the shape of the alveoli. A lower ratio indicates a simple, more rounded struture (i.e. less septa protruding into airspace).

The internal surface area of the lung available for respiratory exchange was calculated using the formula (4 x lung volume)/Lm. These data were normalized to 100g of body weight and used as specific internal surface area (SISA).

Airway Morphometry

Airway layer fractional areas were identified and calculated by the same image-analysis system as the alveoli. Airways cut obliquely, as defined by a circularity (4π x area/perimeter) below 0.7 or above 1.3, were eliminated from analyses. The resulting number of circularly cut small airways averaged 10 per animal. Only small airways with a circumference less than 500 μm, as defined by the total airway wall perimeter, were measured in this study.

Airways projected from the microscope to the computer screen consisted of a lightly stained epithelial layer (EL) a dark elastin band located beneath the EL, and a smooth muscle layer (SML) containing smooth muscle cells, extreacellular collagen, and elastin. The measurement for each airway was accomplished in two steps. The area (A) of the total airway wall (TW) consisting of EL and SML was measured first. The epithelial basement membrane (BM) was defined using the mouse, separating the EL from the SML. AEL was then measured. ASML was calculated as TW-AEL. Each area measurement was normalized to the length of the epithelial BM.

F. IMMUNOCYTOCHEMISTRY (ICC)

Lung Preparation

Lungs from various aged rats were fixed *in situ* with 10% neutral buffered formalin for 2 hours and then removed *en bloc* from the chest. They were then immersed in formalin for at least 24 hours before slicing. Lungs were embedded in paraffin and sliced as they were for morphometry (above). Individual slices were allowed to adhere to APTEX coated slides and ICC for 5-LO was performed.

Immunocytochemistry

Slides with the lung slices were placed in a xylene bath for 5 minutes. This was reapeated for a second 5 minutes and the slides were then put into a series of ethanol baths. They were placed in 100% ethanol for 3 minutes (repeated once) followed by a 3 minute 95% ethanol bath and then a 3 minute 80% ethanol bath. The slides were then rinsed in distilled H_2O for 3 minutes.

6

The slides were then laid flat in a moist box and enough 3% Triton-X was dropped onto each slide (using a Pasteur pipette) to cover the slices. The box was sealed and the samples were left at room temperature for 5 minutes. This was followed by a 3 minute PBS rinse. Then proteinase-K (100 µg/ml in buffer: 1M Tris HCl, 0.5 M EDTA, in DEPC H_2O) was added to the slices and was allowed to incubate at 37°C for 30 minutes. The reaction with proteinase-K was stopped with 0.5% glycine (in 1M Tris HCl, 1M NaCl, DEPC H_2O) for 5 minutes at room temperature. Then the slides were washed in PBS for 5 minutes with 3 changes of PBS.

Endogenous peroxidase was blocked by adding enough 5% H_2O_2 (in methanol) to cover the slices. This was left at room temperature for 10 minutes and was followed by a PBS wash for 5 minutes (with 3 changes of PBS).

After removing as much PBS as possible, 1 drop of normal goat serum (20% in PBS) was added to the slices for 15 minutes at room temperature. The slides were then rinsed in PBS for 3 minutes and as much PBS was removed as possible without letting the slides dry out.

The primary antibody (Pab) for 5-LO was a gift from Merck-Frosst. 5-LO was a rabbit polyclonal raised against native purified human leukocyte 5-lipoxygenase and according to the manufacturer, successfully recognizes rat and human 5-lipoxygenases.

The Pab was diluted in 1% BSA in PBS and was used at a 1:50 dilution. It was added for 4 hours at 4°C. The slides were then washed in PBS for 5 minutes (3 changes) and the secondary antibody (2ab) was added. The 2ab was goat anti-rabbit and conjugated to biotin (from Sigma Chemical Co.) and were diluted 1:20 in 1% BSA. It was allowed to bind to the Pab on the slides for 30 minutes at room temperature, followed by a PBS wash for 5 minutes (3 changes). Extravidin conjugated to peroxidase (Sigma) was diluted in PBS to a 1:20 dilution and was added for 30 minutes at room temperature. This was also followed by a 5 minute PBS wash. Diaminobenzadine was added resulting in a color reaction. It was prepared according to the manufacturer's instructions (Sigma), in distilled H_2O and added to the slides for a very short period (2-4 minutes) until the color reaction was adequate. The slides were then washed in H_2O for 2 minutes and then put through increasing concentrations of ethanol (50% for 3 minutes, 80% for 3 minutes, 100% for 3 minutes) followed by a xylene bath for at least 2 minutes.

Slices were then covered with Permount (Sigma) and a coverslip and allowed to dry before analysis. Cells obtained from BALF were used as positive controls. Negative controls were 14 day old rat lungs from which the Pab was ommitted.

G. PULMONARY FUNCTION

Pulmonary resistance (R_L) was measured in 5 week old anaesthetized (urethane, 1.0-1.75 mg/kg; Sigma) ventilated rat pups. The trachea was canulated with polyethylene tubing 2.5cm long (Intramedic PE 205; Becton Dickson, Rutherford, NJ). Artificial ventilation was accomplished by attaching the cannula to a modified Harvard small-animal respiration pump (tidal volume, 10-12 ml/kg; 40 breaths/min) (Harvard Aparatus Co., South Natick, MA, USA). The bronchoconstrictor, methacholine (0.2 to 20 µg/kg in physiologic saline), was administered intravenously via a 24-guage, three-quarter inch catheter (Insulyte-W; Becton Dickson) inserted into the femoral vein. Resistance values for each breath were measured using the computer-directed View-Dac data acquisition system, which computes the resistance from the flow rate and the pressure (29).

After tracheal cannulation and intravenous catheterization, the spontaneously breathing animals were attached to the ventilator. Baseline R_L was determined after saline was administered and was calculated from the mean of 10 breaths. Then the first dose of methacholine (0.2 µg/kg) was administered, and the peak R_L value was recorded. The animal was removed from the ventilator approximately 30 to 40 breaths after administration of the dose and was allowed to recover for 2 minutes before the procedure was repeated again with the next dose. Resistance changes after methacholine injections were expressed as the percentage of the peak resistance relative to the baseline immediately before injection [(peak R_L/baseline R_L) x 100%). A dose-response curve was generated for each animal, and the EC_{200} was calculated. The EC_{200} is the concentration of methacholine required to increase R_L to 200% of the value measured after saline administration (baseline). We considered baseline values to be 100%.

H. STATISTICS

Experimental data were analyzed for significant differences by one-factor analysis of variance except for data from methacholine dose-response curves which were analyzed by using repeated measures analysis of variance. After discussions with a biostatistician, we assumed that each pup would be treated as an independent observation within the whole population rather than a replicate within a litter. The reasons for this are that litter differences within an experiment were minimal due to cross-lactation, the equal number of pups/litter (12 pups), the age of pups at analysis, and inbreeding. Each experimental group is compared to its own control and not to groups from other experiments. Duncan's New Multiple Range post-hoc test was used to determine differences between groups when a significant F-value was obtained. Significance was achieved at $p \leq 0.05$. Data are expressed as mean ± standard deviation except for Figure 16 where standard error is used to improve clarity of the figure.

IV. RESULTS

A. LEUKOTRIENES ARE INDICATED AS MEDIATORS OF HYPEROXIA-INHIBITED ALVEOLARIZATION IN NEWBORN RATS.

(Portions of these results have been published as part of the paper: Leukotrienes are indicated as mediators of hyperoxia-inhibited alveolarization in newborn rats. Boros V, Burghardt J.S., Morgan C.J., and Olson D.M., Am J Physiol 272, 1997:L433-L441. My contribution to this paper was equal to that of Dr. Boros.)

Objectives:

1) To examine the effect of hyperoxia on LT production by the lungs of rat pups.
2) To examine the effect of LT inhibition on hyperoxia-induced changes in lung parenchymal structure in neonatal rat pups using Wy-50,295 (5-LO inhibitor and LTD_4 receptor antagonist) and MK-0591 (FLAP inhibitor).

Hypotheses:

1) Hyperoxic exposure from days 4-14 causes the lungs to produce elevated LT levels.
2) Inhibition of LT production from days 3-14 prevents damage caused by hyperoxic exposure from days 4-14.

Animals:

Group	Oxygen Exposure	LT Inhibition
RA + V	21% from birth to day 14	Vehicle (H_2O + Tween 80 in a 4:1 ratio) sc 1x daily from days 3-14
RA + WY-50,295	21% from birth to day 14	WY-50,295 30mg.kg, sc 1x daily from days 3-14
RA + MK-0591	21% from birth to day 14	MK-0591 20 mg/kg, sc 1x daily from days 3-14
O_2 + V	>95% from day 4-14	Vehicle (H_2O + Tween 80 in a 4:1 ratio) sc 1x daily from days 3-14
O_2 + WY-50,295	>95% from day 4-14	WY-50,295 30mg/kg, sc 1x daily from days 3-14
O_2 + MK-0591	>95% from day 4-14	MK-0591 20 mg/kg, sc 1x daily from days 3-14

Measurements:

Eicosanoid levels	day 14
Parenchymal morphometry	day 14
BAL	day 12 or 13
wet/dry lung weight ratios	day 12 or 13

Results:

Eicosanoid Production

LT production by lung slices over 30 minutes is shown in Fig. 3. Peptido-LT output was significantly ($p<0.05$) elevated in both O_2 + V groups after the 10 day O_2 exposure (FIg. 3, A and B) compared with their respective controls. As Fig. 3A shows, Wy-50,295 signifcantly ($p<0.05$) inhibited O_2-induced LT output. Peptido-LT output from air-exposed WY-50,295-treated animals decreased; however, it was not significantly different from RA + V animals. Figure 3B shows that MK-0591 inhibited peptido-LT production from both air and O_2-exposed animals; however, peptido-LT levels in the RA + MK-0591 group were not statistically significantly different from the RA + V group. Peptido-LT output from O_2 + V animals was significantly ($p<0.01$) higher than that of the three other groups, including O_2 + MK-0591 (Fig. 3B).

The 6-keto-$PGF_{1\alpha}$ output, a stable metabolite of PGI_2 (prostacyclin), was measured to detect a possible arachidonic acid shunt to the cyclooxygenase pathway because of 5-LO inhibition. We detected significantly ($p<0.05$) increased 6-keto-$PGF_{1\alpha}$ output from all four O_2-exposed groups compared with their respective air-exposed controls (Fig. 4, A and B). The 6-keto-$PGF_{1\alpha}$ production was neither elevated nor diminished because of Wy-50,295 or MK-0591 administration, indicating that neither shunting of arachidonic acid to, nor inhibition of the cyclooxygenase pathway occurred.

Acute Lung Injury

There were no significant differences between the groups in body weight or lung volume at the end of the experimental period (data not shown). Body weights and lung volumes were smaller for all four groups in the MK-0591 experiment compared to the animals in the Wy-50,295 experiment. This is due to the fact that litter sizes were kept larger for a longer period of time during the MK-0591 experiment, i.e. fewer animals were removed on days 12 and 13 for wet/dry weight determinations or BAL, making the litters larger at day 14.

The ELW, as indicated by lung tissue wet/dry weight ratios, was significantly elevated in both O_2+V and O_2+drug groups ($p<0.01$) compared to the air groups (Figure 5A & B). Neither Wy-50,295 nor MK-0591 diminished lung edema caused by oxygen exposure. ELW was similar in the RA+V and RA+drug lungs. In a trend similar to that of ELW, BAL fluid protein levels (Figure 6A & B) were increased in all oxygen-exposed groups compared to air groups ($p<0.01$). Neither drug diminished protein levels.

Morphometry

Subjective observations under the light microscope suggested that O_2 exposed animals had fewer and enlarged alveoli and alveolar ducts compared to air controls (Figure 7B and C). Their architecture was similar to 3 day old animals (Figure 5A). RA+Wy-50,295 lungs showed increased tissue mass and the alveolar septa appeared thick and hypercellular (Figure 7D). O_2+Wy-50,295 animals had lung architecture similar to air treated animals. They had an increased amount of parenchymal tissue and more and smaller air-space units compared to O_2+V pups. The alvelolar septae appeared to be markedly thicker (Figure 7E). RA+ MK-0591 were indistinguishable from RA+V lungs (Figure 8C & A). O_2+MK-0591 animals had a markedly improved structure compared to O_2+V animals without any changes in septal thickness (Figure 8D & B).

The comparative lung morphometric findings for Wy-50,295 treated and control rat pups before oxygen exposure and after 10 days of hyperoxia are shown in Table 5. The volume density of parenchymal tissue (V_p) of O_2+V pups was significantly lower compared to both air groups and the O_2+Wy-50,295 treated group. Furthermore, the parenchymal tissue volume density showed a significant elevation in RA+Wy-50,295 pups compared to RA+V pups. No difference was noted in alveolar septum thickness (Th_{sept}) between oxygen and air exposed pups. In contrast, both Wy-50,295 groups had increased septal wall thickness compared to vehicle-treated controls. The airspace unit volume ($V_{aspunit}$) of O_2+V animals was similar to that of 3 day old animals, while the airspace volume of RA+V and RA+Wy-50,295 was significantly smaller. Wy-50,295 had no effect on airspace volume in air exposed animals compared to its relative vehicle controls, however, the airspace volume was significantly lower in the O_2+Wy-50,295 group compared to the O_2+V group. The airspace perimeter/area ratio (P/A) followed a similar pattern to that of $V_{asp.unit}$. The ratio was significantly smaller in the O_2+V group, much like the values of the 3 day old animals. The O_2+Wy-50,295 ratio was larger than the O_2+V group and similar to both air groups. A decrease was observed in specific internal surface area (SISA) development of O_2+V animals, which was not observed in the O_2+Wy-50,295 group. There was no difference in alveolar diameter or SISA between the 14 day old air groups.

Table 6 shows the morphometric measurements for MK-0591 and vehicle-treated rats pups. O_2 exposure in this experiment caused a significantly ($p<0.05$) lower V_P in O_2+V pups compared to RA+V, RA+MK-0591, and O_2+MK-0591. MK-0591 caused no effects on tissue density in air exposed animals. As with Wy-50,295, no difference was noted in Th_{sept} between O_2 and air exposed pups. Unlike Wy-50295, MK-0591 caused no increases in Th_{sept}. Lm and $V_{aspunit}$ were increased in the O_2+V group, while the values in the O_2+MK-0591 group were similar to air groups. P/A followed a similar pattern, with O_2 exposure causing a significant decrease which was prevented by MK-0591. The SISA was significantly lower in the O_2+ V group. Again, MK-0591 treatment was able to prevent this decrease.

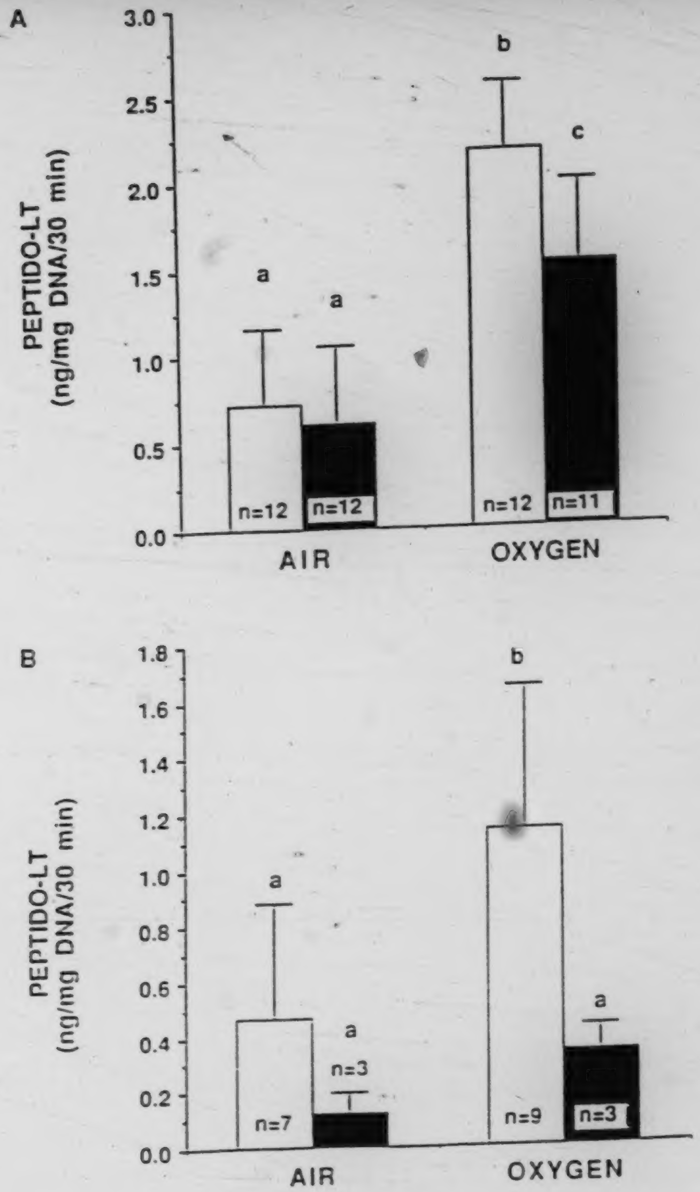


Figure 3. Effect of >95% O_2 exposure from d 4-14, Wy-50295 (30mg/kg daily A) and MK-0591 (20mg/kg daily, B) lung tissue peptido-leukotriene (peptido-LT) output of 14 d old rat pups. Open bars, vehicle; filled bars, Wy-50,295 or MK-0591. O_2 exposure significantly ($p<0.05$) stimulated peptido-LT production. Wy-50295 and MK-0591 were able to reduce peptido-LT output significantly ($p<0.05$) from O_2-exposed animals. Data are expressed as means ± SD. Different letters indicate statistical significance ($p<0.05$) between groups.

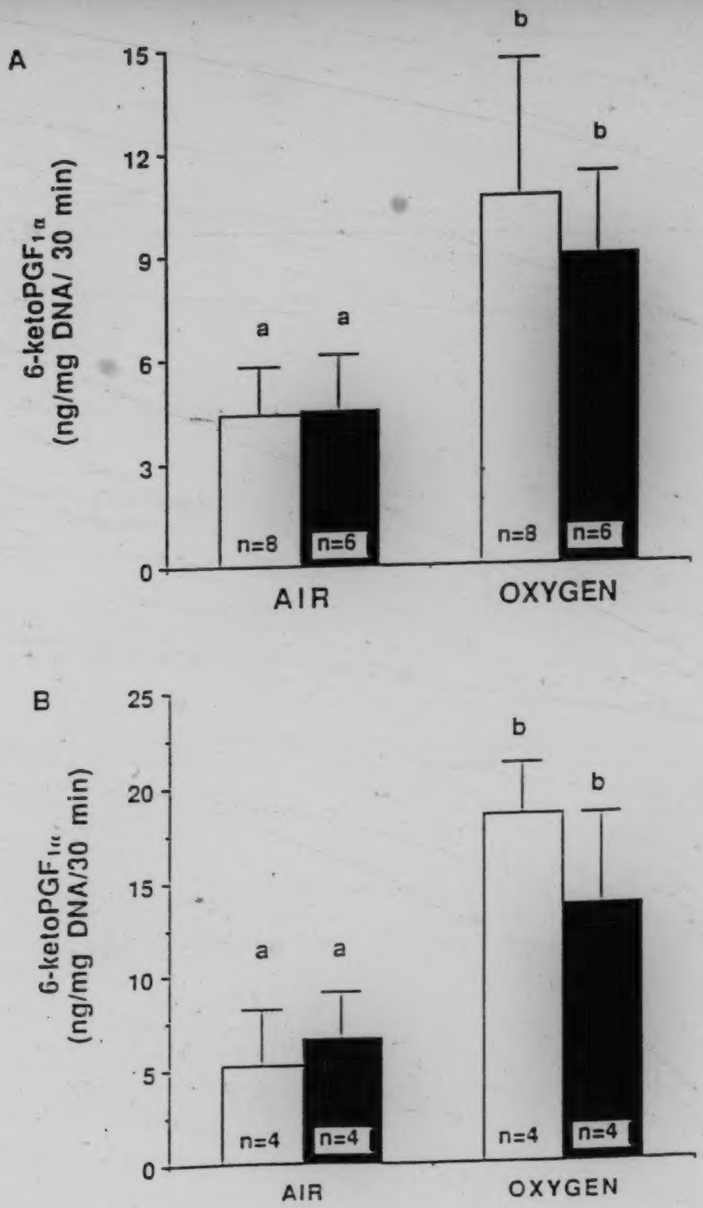


Figure 4. Effect of >95% O_2 exposure from d 4-14, Wy-50295 (30mg/kg daily A) and MK-0591 (20mg/kg daily, B) lung tissue 6-ketoprostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) output of 14 d old rat pups. Open bars, vehicle; filled bars, Wy-50,295 or MK-0591. O_2 exposure significantly ($p<0.05$) stimulated 6-keto $PGF_{1\alpha}$ production, but Wy-50295 or MK-0591 did not influence it. Data are expressed as means ± SD. Different letters indicate statistical significance ($p<0.05$) between groups.

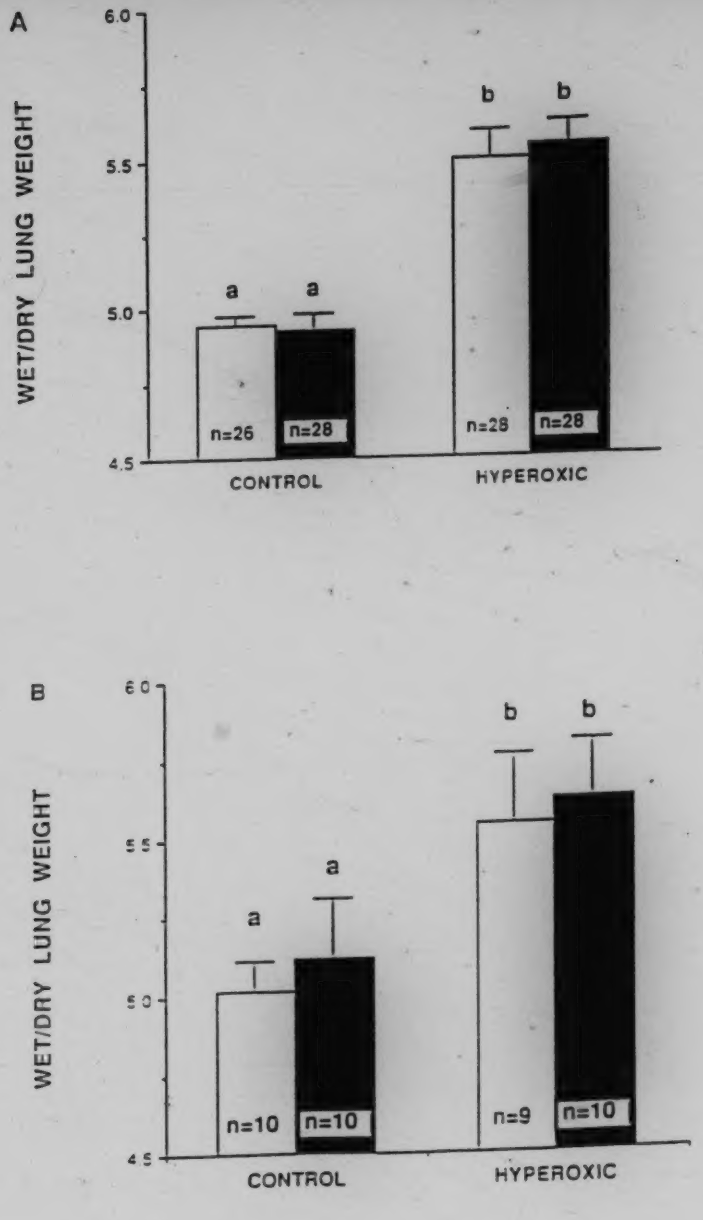


Figure 5. Effect of >95% O_2 exposure from d 4-14, Wy-50295 (30mg/kg daily A) and MK-0591 (20mg/kg daily, B) on wet-to-dry lung weight ratios of 14 d old rat pups. Open bars, vehicle; filled bars, Wy-50,295 or MK-0591. The extravascular lung water content significantly ($p<0.01$) increased in all O_2-exposed groups. Neither Wy-50295 not MK-0591 altered wet-to-dry lung ratios. Data are expressed as means ± SD. Different letters indicate statistical significance ($p<0.05$) between groups.

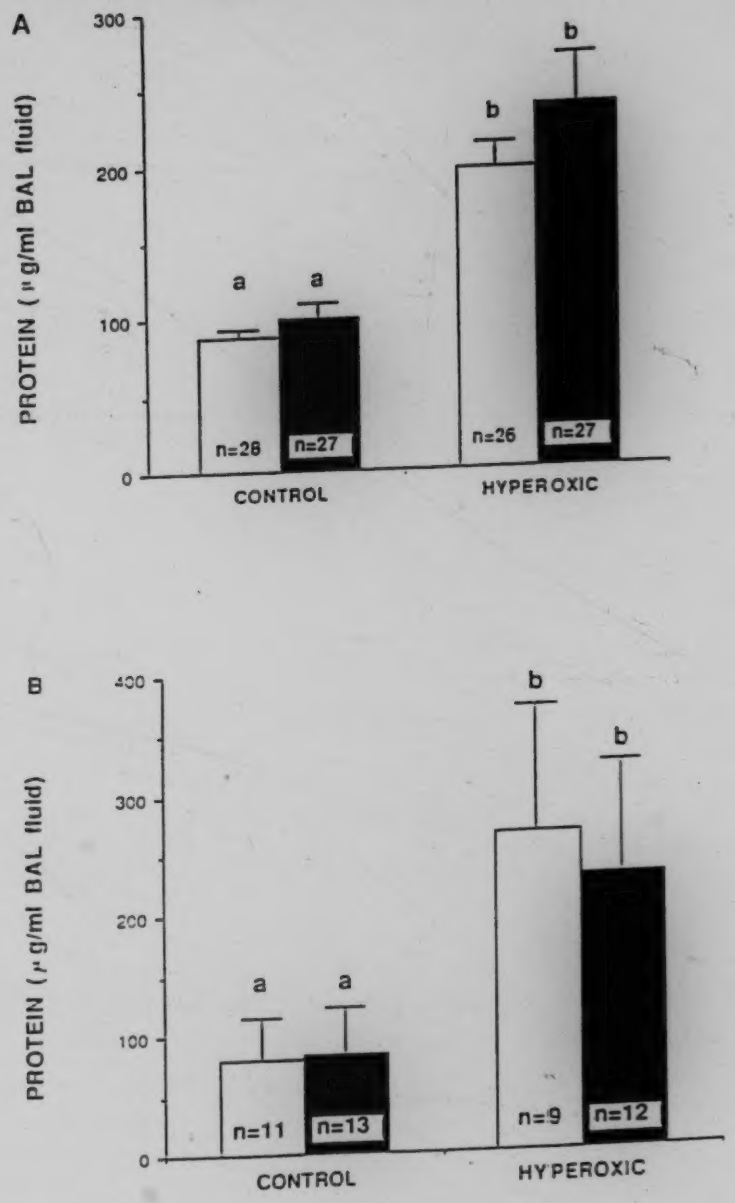


Figure 6. Effect of >95% O_2 exposure from d 4-14, Wy-50295 (30mg/kg daily, A), and MK-0591 (20mg/kg daily, B) on protein content of bronchoalveolar lavage fluid of 14 d old rat pups. Open bars, vehicle; filled bars, Wy-50295 or MK-0591. O_2-exposed pups had significantly higher protein levels ($p<0.01$). Neither Wy-50295 nor MK-0591 altered the protein content. Data are expressed as means ± SD. Different letters indicate significant differences ($p<0.05$) between groups.

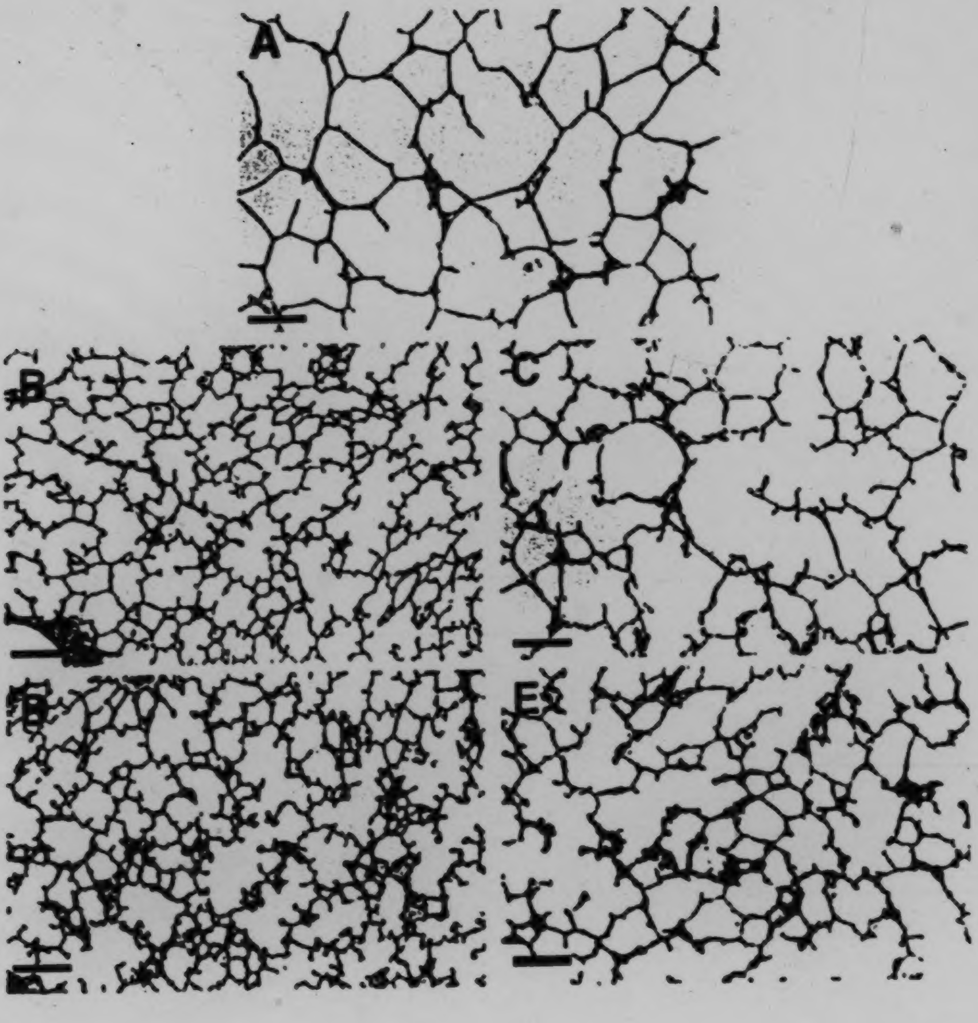


Figure 7. Photomicrographs of rat pup lung parenchyma. **A**: 3d old, saccular stage. **B**: 14d old, 21% O_2 + Vehicle (RA + Vehicle). **C**: 14d old, >95% O_2 from d4-14 + Vehicle (O_2 + Vehicle). **D**: 14d old, 21% O_2 + Wy-50,295 from d3-14 (RA + Wy-50,295). **E**: 14d old, >95% O_2 from d4-14 + Wy-50,295 from d3-14 (O_2 + Wy-50,295). The parenchymal architecture of hyperoxic pups is simpler (fewer and enlarged alveoli; C). The alveolar septae of RA + WY-50,295-treated pups are bulkier; note the apparent hypercellularity (D). Pulmonary structure of O_2 + Wy-50,295 (E) pups contains more and smaller alveoli compared with the O_2 + Vehicle (C) lung. Bar=100μm.

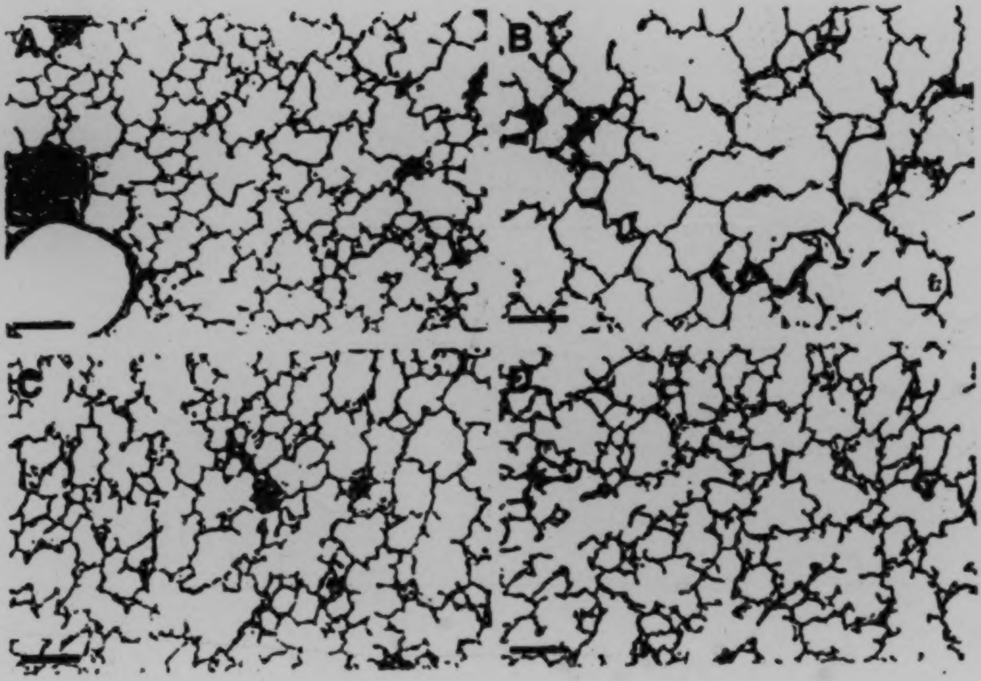


Figure 8. Photomicrographs of lung parenchyma of 14d old rats. A: 21% O_2 + Vehicle (RA + Vehicle). **B**: >95% O_2 from d4-14 + Vehicle (O_2 + Vehicle). **C**: 21% O_2 + MK-0591 from d3-14 (RA + MK-0591). **D**: >95% O_2 from d4-14 + MK-0591 from d3-14 (O_2 + MK-0591). LT synthesis inhibition by MK-0591 (D) prevented the inhibition of alveolarization seen in O_2 + Vehicle lungs (B). Bar=100μm.

Table 5. The effects of hyperoxic exposure and Wy-50,295 on lung morphometry of 14 day old rat pups.

GROUP	n	$V_{asp\ unit}$ ($\mu m^3 x10^5$)	P/A	Vp (%)	Th sept (μm)	SISA ($cm^2/gx10^3$)
3 day	5	2.23 ± 0.64^a	0.67 ± 0.10^a	21.0 ± 2.1^a	0.84 ± 0.05^a	4.59 ± 0.43^a
14 day air+vehicle	6	0.81 ± 0.23^b	0.88 ± 0.07^b	22.5 ± 1.7^b	0.63 ± 0.08^b	4.99 ± 0.64^a
14 day air+Wy50295	6	0.88 ± 0.20^b	0.90 ± 0.09^c	25.5 ± 1.8^c	0.75 ± 0.04^a	5.26 ± 0.56^a
14 day O_2+vehicle	6	1.84 ± 0.84^a	0.70 ± 0.04^a	19.3 ± 2.3^a	0.68 ± 0.10^b	3.85 ± 0.64^b
14 day O_2+Wy50295	6	0.99 ± 0.26^a	0.80 ± 0.07^b	23.6 ± 2.6^b	0.76 ± 0.06^a	4.82 ± 0.55^a

Values are expressed as means±SD. Different superscripts indicate significant differences at $p<0.05$. $V_{asp\ unit}$, mean airspace unit volume; P/A, ratio of airspace perimeter to area; Vp, volume density of parenchymal tissue; Th sept, mean septal thickness; SISA, specific internal surface area.

Table 6. The effects of hyperoxic exposure and MK-0591 on the lung morphometry of 14 day old rat pups.

GROUP	n	$V_{asp\ unit}$ ($\mu m^3 x10^5$)	P/A	Vp (%)	Th sept (μm)	SISA ($cm^2/gx10^3$)
14 day air+vehicle	6	0.78 ± 0.19^a	0.89 ± 0.04^a	22.9 ± 1.4^a	0.67 ± 0.04^a	5.18 ± 0.64^a
14 day air+MK0591	6	0.82 ± 0.29^a	0.90 ± 0.09^a	23.6 ± 3.3^a	0.69 ± 0.07^a	5.05 ± 0.49^a
14 day O_2+vehicle	6	1.48 ± 0.55^b	0.74 ± 0.09^b	19.8 ± 3.4^b	0.67 ± 0.08^a	4.31 ± 0.34^b
14 day O_2+MK0591	6	0.85 ± 0.28^a	0.90 ± 0.07^a	24.1 ± 2.6^a	0.73 ± 0.06^a	4.94 ± 0.36^a

Values are expressed as means±SD. Different superscripts indicate significant differences at $p<0.05$. $V_{asp\ unit}$, mean airspace unit volume; P/A, ratio of airspace perimeter to area; Vp, volume density of parenchymal tissue; Th sept, mean septal thickness; SISA, specific internal surface area.

B. OXYGEN HAS AN INDIRECT EFFECT ON ALVEOLAR DEVELOPMENT THAT IS MEDIATED BY LEUKOTRIENES

Objectives:

1) To determine whether or not there is a critical period during alveolarization for the O_2 effect on the developing lung
2) To determine whether or not a critical period for O_2 can be connected with a critical period for leukotriene effects.

Hypotheses:

1) Oxygen exposure from days 4-9 will have a more profound effect on alveolar development than exposure from days 9-14.
2) Leukotriene inhibition (MK-0591) from days 4-9 in animals exposed to hyperoxia from days 4-14 will prevent hyperoxia-induced damage (measured on day 14), while inhibition from day 9-14 will have no effect.

Animals:

Group	Oxygen Exposure	LT Inhibition
RA	21% from days 4-14	Vehicle (H_2O + Tween 80 in a 4:1 ratio) sc 1x daily from days 3-14
RA + MK-0591 (4-9)	21% from days 4-14	MK-0591, 20 mg/kg, sc 1x daily from days 4-9
RA + MK-0591 (9-14)	21% from days 4-14	MK-0591, 20 mg/kg, sc 1x daily from days 9-14

O_2 (4-14)	>95% from days 4-14	Vehicle (H_2O + Tween 80 in a 4:1 ratio) sc 1x daily from days 3-14
O_2 (4-9)	>95% from days 4-14	Vehicle (H_2O + Tween 80 in a 4:1 ratio) sc 1x daily from days 3-9
O_2 (9-14)	>95% from days 4-14	Vehicle (H_2O + Tween 80 in a 4:1 ratio) sc 1x daily from days 9-14
O_2 + MK-0591(4-9)	>95% from days 4-14	MK-0591, 20 mg/kg, sc 1x daily from days 4-9
O_2 + MK-0591 (9-14)	>95% from days 4-14	MK-0591, 20 mg/kg, sc 1x daily from days 9-14
O_2 + MK-0591 (4-14)	>95% from days 4-14	MK-0591, 20 mg/kg, sc 1x daily from days 4-14

Measurements:

Leukotriene levels	days 9 and 14
Parenchymal morphometry	day 14

Results:

Leukotriene Production

Oxygen exposure from days 1-4 caused a signficant increase in peptido-LT production at day 4 compared to air controls. These levels returned to normal by days 9 and 14 (Figure 9). Oxygen exposure from days 4-9 causes a significant ($p<0.05$) increase in the amount of peptido-LT produced by rat pup lungs measured on day 9 compared to air controls (Figure 10). By day 14, peptido-LT levels return to control levels in these pups. On day 14 pups exposed to O_2 from days 4-14 and 9-14 produce signifcantly ($p<0.05$) higher levels of peptido-LTs than air controls (Figure 10).

Exposure to oxygen from days 4-9 caused a significant increase in LT produced by the lung on day 9 (Figure 11) which was signficantly reduced by MK-0591. By day 14 the levels returned to normal in these pups, while pups exposed from days 9-14 had increased LT levels (Figure 11). Once again, inhibition of peptido-LT production was significant.

Morphometry

Effects of differential exposure to hyperoxia

Representative photomicrographs of lung parenchyma from each of the experimental groups are depicted in figure 12. Compared to air animals, the rats exposed to hyperoxia from days 4-14 have larger and more simplified alveolar airspaces. The days 4-9 exposed animals exhibit parallel changes in alveolar structure, although to a lesser extent than the changes noted in the day 4-14 group. In contrast, the days 9-14 are similar to the air animals, with respect to alveolar size and shape.

These visual impressions are correlated and quantified by the morphometric data (Table 7). Exposure to >95% O_2 from days 4 to 14 causes an increase in Lm, an estimate of alveolar diameter, and an associated increase in the calculated airspace volume, $V_{aspunit}$. The pups exposed from day 4 to 9 manifest elevated Lm and $V_{aspunit}$ compared to air animals. However, the increases seen in this latter group are significantly lower than those observed for the days 4-14 group. Four day exposure prior to day 4 and following day 9 has no detectable effect on these alveolar size related parameters. The intergroup differences seen with respect to Lm and $V_{aspunit}$ are mirrored by the P/A ratio. The animals exposed from day 4 to 14 evidence simplified alveoli, as indicated by a decreased P/A ratio. The day 4-9 group also have a decreased P/A ratio, but again this decrease is not as great as that which is associated with the day 4 to 14. Animals exposed from day 9 to 14 have a P/A ratio comparable to the air animals.

In contrast to the parameters described above, significant alterations in SISA are only seen in the group exposed from day 4 to 14. The pups in this group have a decrease in lung surface area.

Some different trends are observable with respect to measures of parenchymal tissue and septal thickness. The relative amount of lung parenchymal tissue, as assessed by V_p, is similarly reduced, compared to air, in the 4-9 and 4-14 groups. O_2 from day 9 to 14 results in lower, but not significantly different, V_p. Meanwhile, Th_{sept} is significantly lower than air animals in the 4-9 and 9-14 groups. The 4-14 animals are transitional between the air animals and these two groups, showing no significant differences when compared to air, but also no significant differences compared to the 4-9 and 9-14 groups.

Effects of leukotriene synthesis inhbition

Photomicrographs showing 14 day oldrat pups exposed to>21% O_2 (A) or >95% O_2 from days 4-14 (B) with administration of MK-0591 (20 mg/kg, sc, once daily) or vehicle from days 4-9, 9-14, or 4-14 are shown in Figure 13. It is obvious from these photos that oxygen vehicle animals have much larger and simplified alveoli than air vehicle animals. This study confirmed previous studies that the administration of MK-0591 from days 3-14 prevents damage caused by hyperoxia from days 4-14 (photo not shown). Also evident from these photos is the prevention of this event in hyperoxic animals given MK-0591 from days 4-9 (Figure 13C) or 9-14 (Figure 13 D), whose alveolar structure is similar to that of the air vehicle group. The drug has no effect in air exposed animals (photos not shown).

The comparative lung morphometric findings for MK-0591- or vehicle-treated air and hyperoxic animals pups at day 14 is shown in Table 8. The volume density of parenchymal tissue (V_p) of O_2+Vehicle pups was significantly lower than that of all air groups as well as all three O_2+ MK-0591 groups. Alveolar septa were significantly thicker in all hyperoxia-exposed animals (regardless of MK-0591 administration compared to the air animals except for the air + MK-0591 (9-14) group which was statistically similar. As with previous studies, hyperoxia alone had a significant alveolar enlarging effect as indicated by airspace unit volume ($V_{aspunit}$) compared to air animals. The $V_{aspunit}$ was not significantly different in hyperoxia-exposed pups administered MK-0591 at anytime, compared to air pups. The airspace perimeter/area ratio (P/A) was significantly smaller in the O_2 + Vehicle group compared to all air groups. Statistical analysis revealed that while the P/A of all three of the O_2 + MK-0591 groups were not signficantly different from the air groups, they were also not significantly different from the O_2 + Vehicle group. The trend was towards and increase in P/A compared to O_2 + Vehicle, however, a decrease was observed in SISA development in O_2 + Vehicle pups, compared to air pups, which was not observed in the O_2 + MK-0591 (4-9) or O_2 + MK-

0591 (9-14) groups. While SISA was higher in the O_2 + MK-0591 (4-14) pups, it did not reach statistical significance (p=0.07).

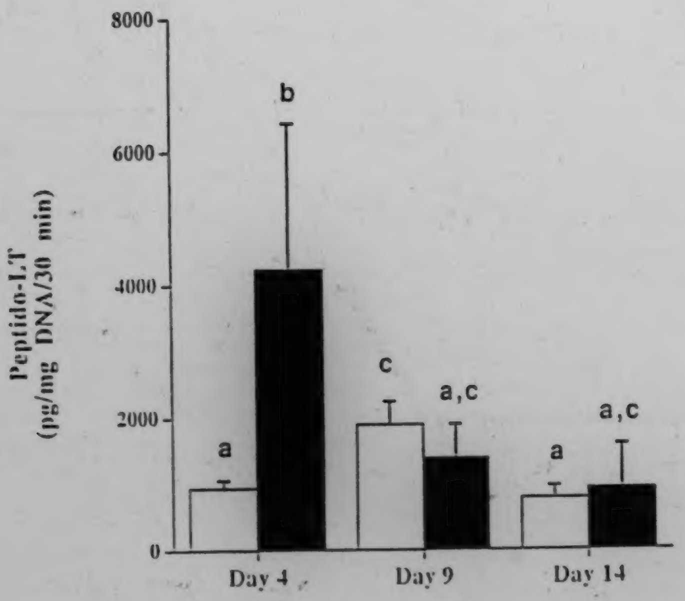


Figure 9. Effects of >95% O_2 exposure from d 1-4 on lung tissue peptido-leukotriene (peptido-LT) output from 4, 9, and 14 d old rat pups. Open bars, air; filled bars, hyperoxia. O_2 exposure from d 1-4 significantly ($p<0.05$) stimulated peptido-LT production in 4 d old rat pups. Data are expressed as means ± SD. n=6 for each group. Different letters indicate significant differences ($p<0.05$).

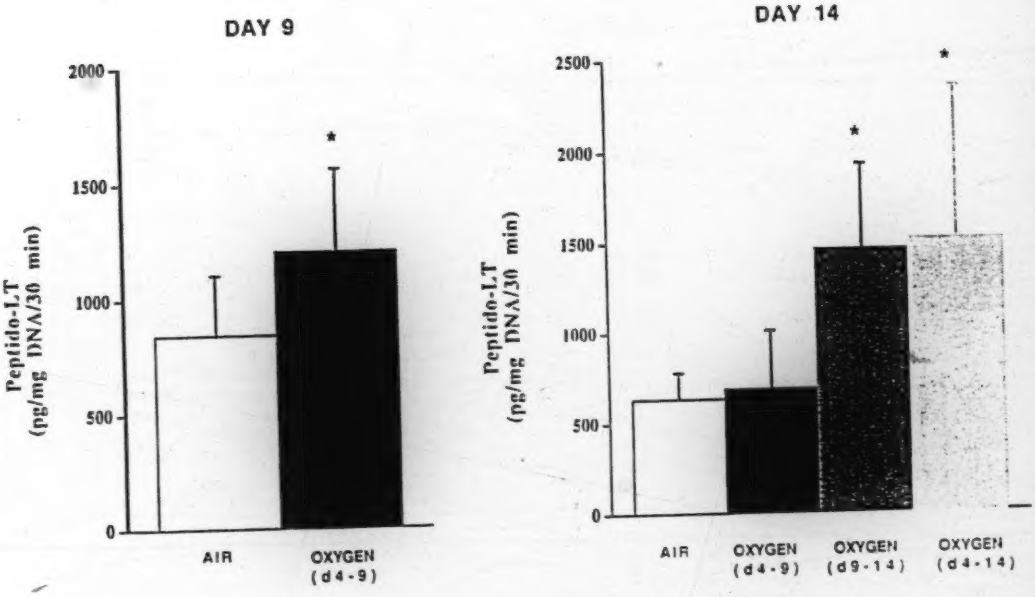


Figure 10. Effects of >95% O_2 exposure from d 4-9, 9-14, or 4-14 on lung tissue peptido-leukotriene (peptido-LT) output from 9 and 14 d old rat pups. O_2 exposure from d 4-9 significantly ($p<0.05$) stimulated peptido-LT production at d 9, which returned to normal by d 14. O_2 exposure from d 9-14 or 4-14 resulted in significantly higher peptido-LT production at d 14 compared to air controls. Data are expressed as means ± SD. n=6 for each group. * indicates significant differences ($p<0.05$).

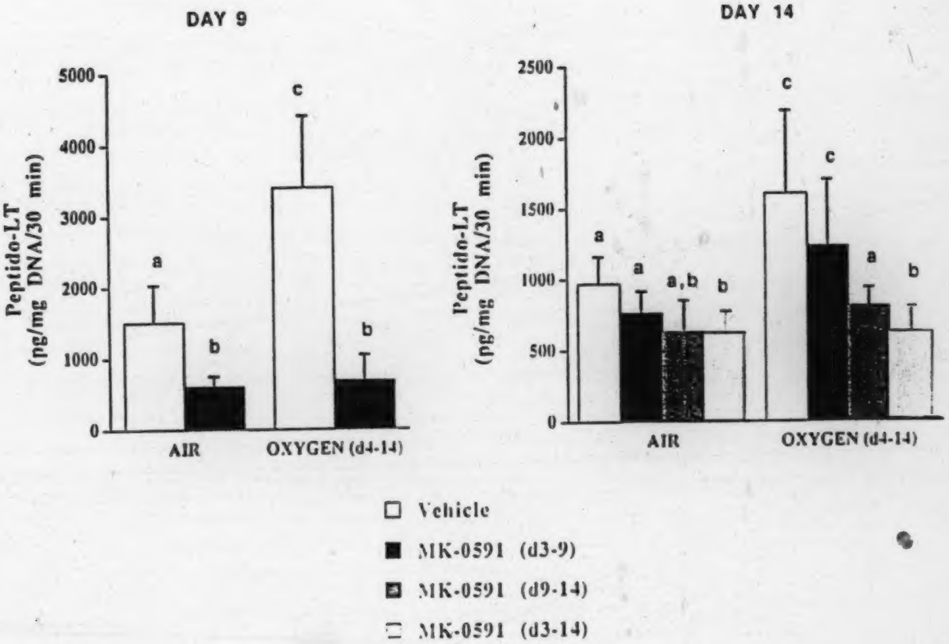


Figure 11. Effects of >95% O_2 exposure from day 4-14 with or without MK-0591 (20mg/kg once daily) from d 3-9, 9-14, or 3-14 on lung tissue peptido-leukotriene (peptido-LT) output from 9 and 14 d old rat pups. O_2 exposure from d4-14 significantly ($p<0.05$) increased peptido-LT production on d 9 and 14. MK-0591 administration from d 3-9 significantly ($p<0.05$) inhibited peptido-LT production on d 9 in O_2 exposed pups which was reversed by d 14. MK-0591 administration from d 3-14 or 9-14 significantly ($p<0.05$) inhibited peptido-LT production at d 14. Data are expressed as means ± SD. n=6 for each group. Different letters indicate significant differences ($p<0.05$).

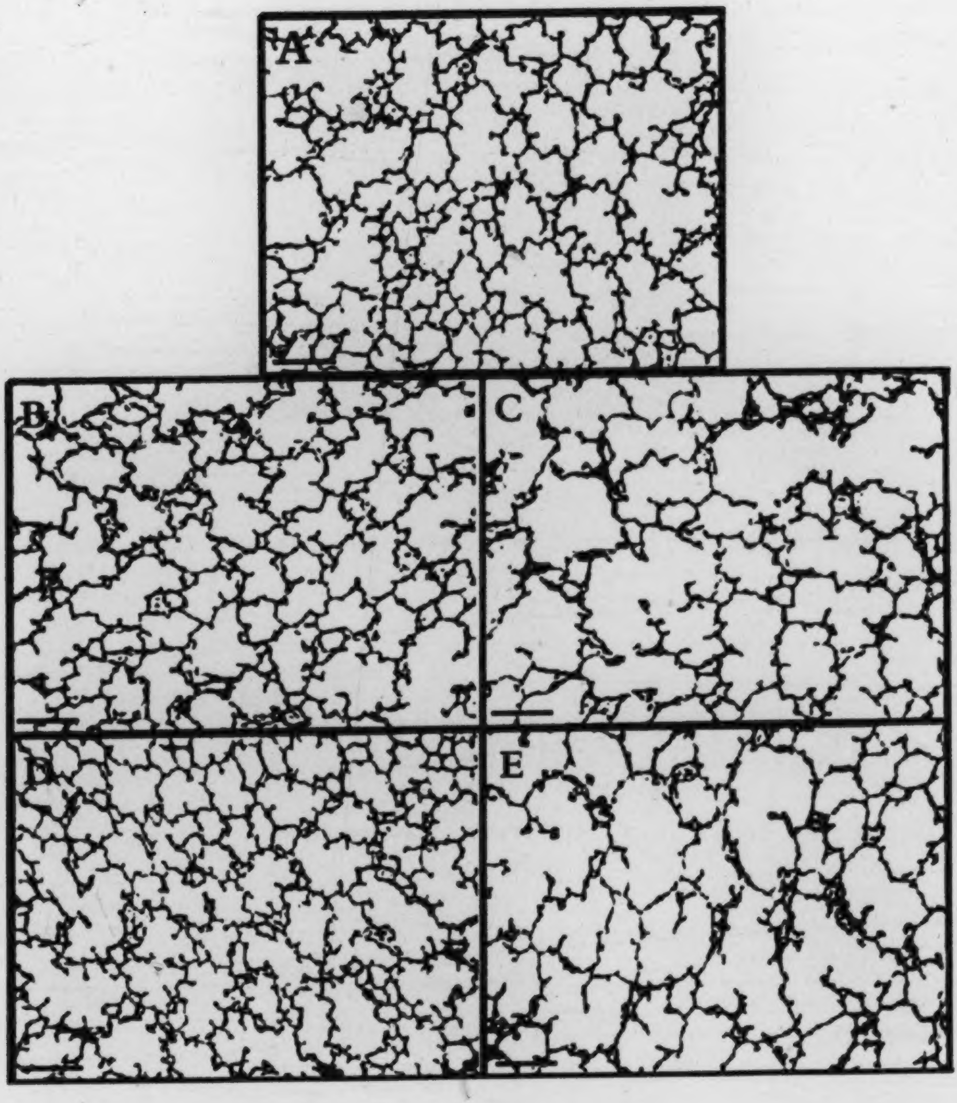


Figure 12. Photomicrographs of lung parenchyma of 14d old rats. **A**: 21% O_2. **B**: >95% O_2 from d1-4. **C**: >95% O_2 from d4-9. **D**: >95% O_2 from d9-14. **E**: >95% O_2 from d4-14. Parenchymal architecture of hyperoxic lungs exposed from d4-9 and d4-14 is simpler (fewer and enlarged alveoli; C and E). Hyperoxic exposure from d1-4 or d9-14 had little effect. Bar=100µm.

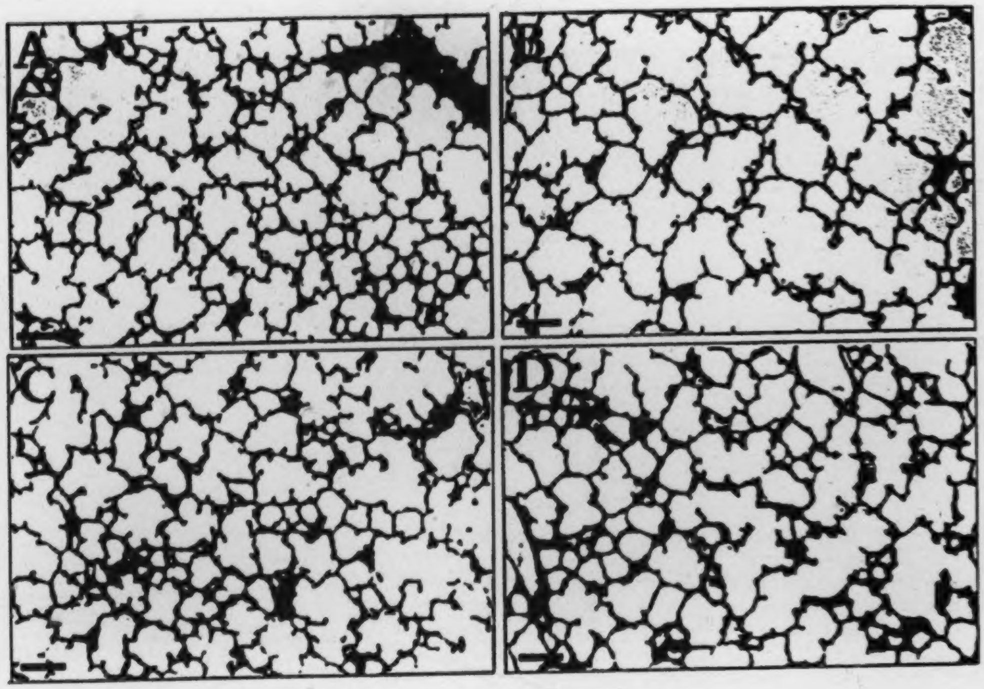


Figure 13. Photomicrographs of lung parenchyma of 14d old rats. **A**: 21% O_2 + Vehicle. **B**: >95% O_2 from d4-14 + Vehicle (O_2 + Vehicle). **C**: >95% O_2 from d4-14 + MK-0591 from d3-9 (O_2 + MK-0591 (3-9)). **D**: >95% O_2 from d 4-14 + MK-0591 from d9-14 (O_2 + MK-0591 (9-14)). LT synthesis inhibition by MK-0591 from d3-9 (C) or 9-14 (D) prevented the inhibition of alveolarization observed in O_2 + Vehicle lungs. MK-0591 has no effect in air animals (not shown). Bar=100μm.

Table 8. The effects of hyperoxic exposure from d4-14 with or without MK-0591 (20mg/kg, sc, once daily, d3-9, d9-14, or d3-14) on lung morphometry of 14 day old rat pups.

GROUP	n	$V_{asp\ unit}$ ($\mu m^3 x 10^5$)	P/A	Vp (%)	Th sept (μm)	SISA ($cm^2/g x 10^3$)
Air +Vehicle	10	0.56 ± 0.27^a	0.94 ± 0.10^a	24.1 ± 2.3^a	0.67 ± 0.03^a	5.97 ± 1.39^a
Air + MK0591(3-9)	6	0.65 ± 0.23^a	0.96 ± 0.04^a	25.5 ± 1.7^a	0.71 ± 0.07^a	6.10 ± 1.03^a
Air + MK0591(9-14)	6	0.63 ± 0.14^a	1.01 ± 0.06^a	25.5 ± 2.9^a	0.74 ± 0.08^b	5.94 ± 0.90^a
Air + MK0591(3-14)	7	0.79 ± 0.29^a	0.90 ± 0.10^a	24.5 ± 2.5^a	0.69 ± 0.07^a	5.05 ± 0.49^a
O_2 + Vehicle	12	1.28 ± 0.54^b	0.82 ± 0.12^b	21.5 ± 3.6^b	0.72 ± 0.06^b	4.56 ± 0.52^b
O_2 + MK0591(3-9)	8	0.89 ± 0.33^a	0.91 ± 0.10^{ab}	25.8 ± 3.1^a	0.78 ± 0.07^b	5.66 ± 0.79^a
O_2 + MK0591(9-14)	7	0.74 ± 0.26^a	0.88 ± 0.04^{ab}	24.5 ± 1.8^a	0.74 ± 0.04^b	5.13 ± 0.83^a
O_2 + MK0591(3-14)	8	0.80 ± 0.26^a	0.89 ± 0.07^{ab}	24.6 ± 2.1^a	0.73 ± 0.06^b	4.94 ± 0.36^b

Values are expressed as means±SD. Different superscripts indicate significant differences at $p<0.05$. $V_{asp\ unit}$, mean airspace unit volume; P/A, ratio of airspace perimeter to area; Vp, volume density of parenchymal tissue; Th sept, mean septal thickness; SISA, specific internal surface area.

Table 7. The effects of hyperoxic exposure during different periods of alveolarization on lung morphometry of 14 day old rat pups.

GROUP	n	$V_{asp\ unit}$ ($\mu m^3 x10^5$)	P/A	Vp (%)	Th sept (μm)	SISA ($cm^2/gx10^3$)
Air	13	0.73±0.21 ^{a}	1.04±0.10 ^{a}	33.7±3.6 ^{a}	0.99±0.12 ^{a}	4.89±0.66 ^{a}
O_2 (1-4)	6	0.68±0.14 ^{a}	1.11±0.05 ^{a}	38.0±1.8 ^{b}	1.11±0.07 ^{b}	4.84±0.35 ^{a}
O_2 (4-9)	15	1.03±0.33 ^{b}	0.96±0.11 ^{b}	29.6±5.4 ^{cd}	0.89±0.16 ^{c}	4.81±0.69 ^{a}
O_2 (9-14)	14	0.63±0.15 ^{a}	1.07±0.06 ^{a}	30.5±2.3 ^{ac}	0.85±0.11 ^{c}	5.26±0.65 ^{a}
O_2 (4-14)	6	1.30±0.18 ^{c}	0.80±0.07 ^{c}	27.1±1.3 ^{d}	0.93±0.04 ^{ac}	4.16±0.37 ^{b}

Values are expressed as means±SD. Different superscripts indicate significant differences at $p<0.05$. $V_{asp\ unit}$, mean airspace unit volume; P/A, ratio of airspace perimeter to area; Vp, volume density of parenchymal tissue; Th sept, mean septal thickness; SISA, specific internal surface area. All n's as indicated except air SISA where n=9.

C. LIPID MEDIATORS IN OXYGEN-INDUCED AIRWAY REMODELLING AND HYPERRESPONSIVENESS IN NEWBORN RATS

(Portions of these results have been published as part of the paper: Lipid mediators in oxygen0induced airway remodelling and hyperresponsiveness in newborn rats. Burghardt J.S., Boros V., Biggs D.F., and Olson D.M. Am J Respir Crit Care Med 154, 1997:837-842.)

Objectives:

1) To characterize both the functional and morphologic changes of airways after chronic oxygen exposure during the newborn period
2) To study the association of these events with leukotrienes in hyperoxia-induced airway remodelling and hyperresponsiveness.

Hypotheses:

1) Hyperoxic exposure from days 4-32 will alter airway wall thickness and cause hyperresponsiveness to methacholine.
2) Leukotriene inhibition (days 3-32) will prevent airway damage caused by hyperoxia.

Animals:

Group	Oxygen Exposure	LT Inhibiton
RA + Vehicle	21% from birth to day 35	Vehicle (H_2O + Tween 80 in a 4:1

		ratio, sc 1x daily, from days 3-14 Methylcellulose 1.5%, po 1x daily from days 15-32)
RA + WY-50,295	21% from birth to day 35	Wy-50,295 30 mg/kg, sc 1x daily from days 3-14 30 mg/kg. po 1x daily from days 15-32
O_2 + Vehicle	>95% from days 4-14 ~65% from days 15-32	Vehicle (H_2O + Tween 80 in a 4:1 ratio, sc 1x daily, from days 3-14 Methylcellulose 1.5%, po 1x daily from days 15-32)
O_2 + WY-50,295	>95% from days 4-14 ~65% from days 15-32	Wy-50,295 30 mg/kg, sc 1x daily from days 3-14 30 mg/kg. po 1x daily from days 15-32

Measurements:

Airway morphometry	day 34
Pulmonary function	day 35
LT levels	day 28
BAL, wet/dry lung weight	day 34

Results:

Eicosanoid production

Peptido-LT production from lung and large airway is shown in figures 14 and 15. After 30 minutes of incubation, peptido-LT production was significantly ($p<0.05$) elevated in the O_2 + Vehicle group from both lung and airway tissue from 2 week old rat pups (figures 14A and 15A). Meanwhile, at 4 weeks, only the airway tissue peptido-LT output showed significant ($p<0.05$) elevation (figure 14B) due to O_2 exposure. The lung tissue peptido-LT output from the O_2 + Vehicle group was similar to the RA + Vehicle group (figure 15B).

At both ages Wy-50,295 significantly ($p<0.05$) inhibited the production of peptido-LTs in the large airway of oxygen-exposed animals. The levels from the O_2 + Wy-50,295 groups were similar to the RA + Vehicle group. Levels were decreased in air-exposed animals, however, they were not found to be statistically significantly different from the RA + Vehicle group (Figures 14A and B).

At 2 weeks, Wy-50,295 was able to decrease the production of peptido-LTs from lung tissue of oxygen-exposed animals but not to normal (air + Vehicle) levels. As with the airway, Wy-50,295 was able to decrease peptido-LT levels at 2 weeks of age but not significantly. In 4 week lung tissue, the peptido-LT production was not influenced by oxygen-exposure, i.e. production in the O_2 + Vehicle group was equal to production in the air + Vehicle group. Wy-50,295 significantly ($p<0.05$) inhibited the LT production in lung tissue in both air and oxygen groups.

Pulmonary function

Baseline R_L values are shown in Table 9. There was no significant difference in baseline R_L among any of the groups although the value for the O_2 + Vehicle group was somewhat higher than the others.

Oxygen-exposure caused a significantly ($p<0.05$) higher airway response to methacholine in 5 week old rat pups as evidenced by a higher resistance change at doses 2-20 µg/kg (Figure 16). Administration of Wy-50,295 effectively prevented the formation of oxygen-induced airway hyperresponsiveness, while it had no effect in air animals. Figure 16 shows that the values for the O_2 + Wy-50,295 group were equal to the RA + Vehicle and RA + Wy-50,295 groups. The same trend was evidenced by the EC_{200} values (Table 9). EC_{200} was significantly ($p<0.05$) lower in the O_2 + Vehicle group compared to all other groups.

ELW

To determine the possible contribution of edema to airway obstruction, ELW was measured. The lungs of oxygen exposed animals were not edematous at 4 weeks of age as evidenced by equal wet/dry lung weight ratios between all four groups (Figure 17).

Airway morphometry

Table 10 shows airway morphometric data of 5 week old rat pups. Oxygen exposure significantly increased the A_{TW} ($p<0.05$) and A_{SML} ($p<0.001$) compared to all other groups. Meanwhile A_{EL} was not affected by oxygen exposure. In O_2 + Wy-50,295 pups, A_{TW} and A_{SML} were equal to the corresponding areas of both air groups.

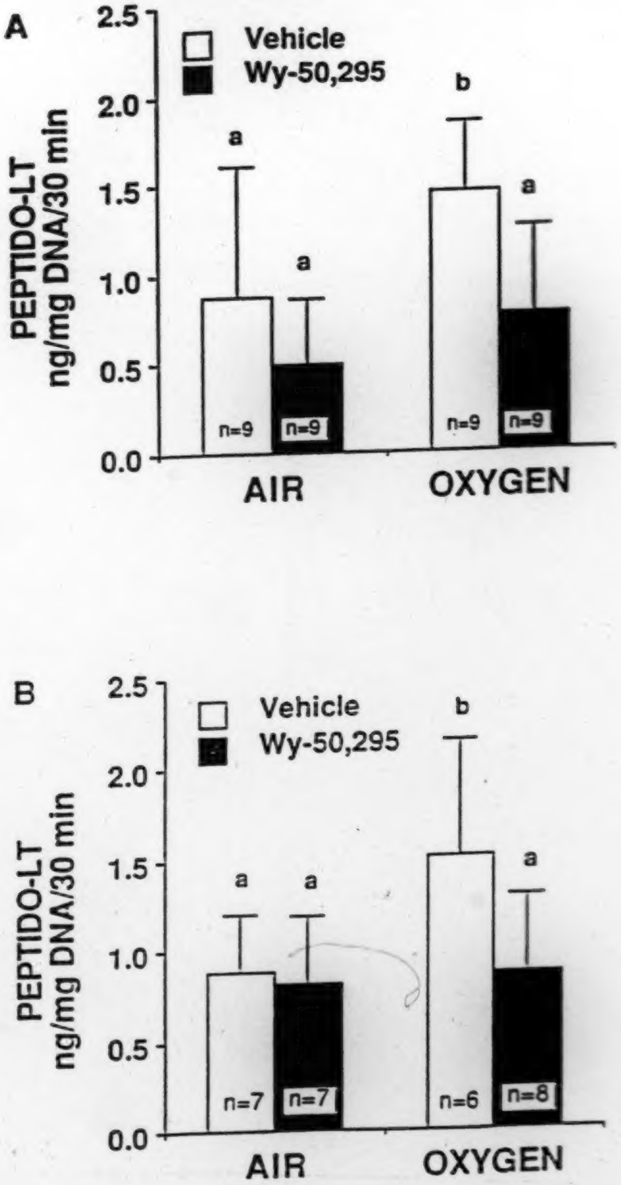


Figure 14. Effect of >95% O_2 exposure and Wy-50295 (30mg/kg daily) on large airway (extrapulmonary airways) peptido-leukotriene (peptido-LT) production of 2 wk old (A) and 4 wk old (B) rat pups. O_2 exposure significantly ($p<0.05$) stimulated peptido-LT production at both ages. Wy-50295 and was able to reduce peptido-LT output significantly ($p<0.05$) in O_2-exposed animals. Data are expressed as means ± SD. Different letters indicate statistical significance ($p<0.05$) between groups.

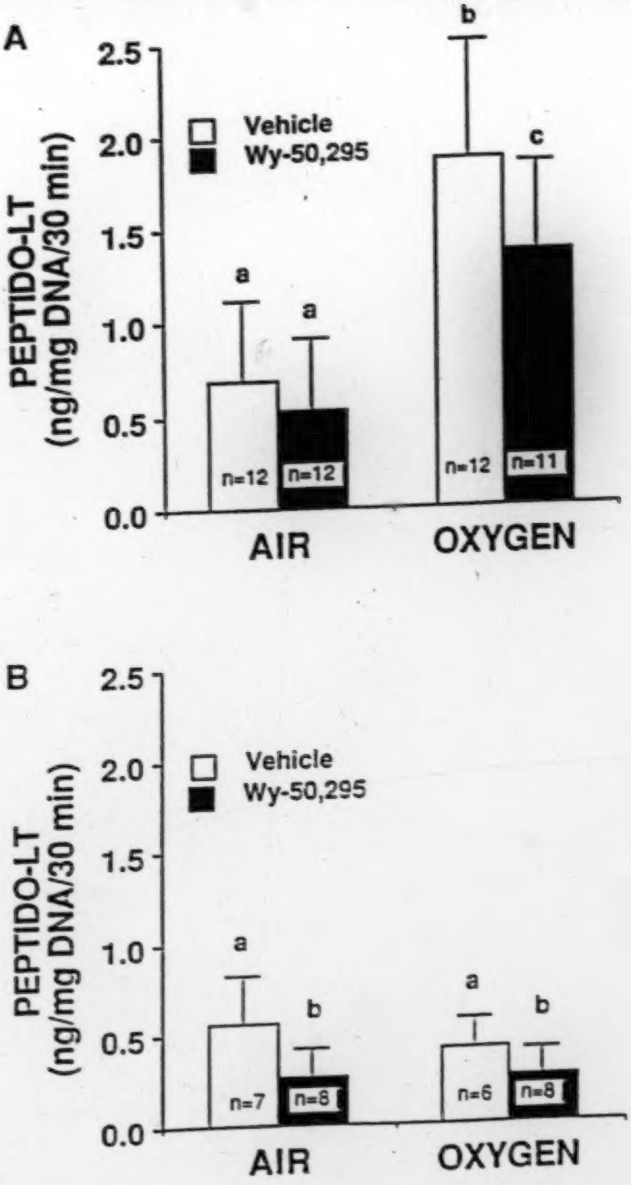


Figure 15. Effect of >95% O_2 exposure and Wy-50295 (30mg/kg daily) on lung peptido-leukotriene (peptido-LT) production of 2 wk old (A) and 4 wk old (B) rat pups. At 2 wk, O_2 exposure significantly increased peptido-LT output. Wy-50295 significantly diminished peptido-LT production of lung tissue. Data are expressed as means ± SD. Different letters indicate statistical significance ($p<0.05$) between groups.

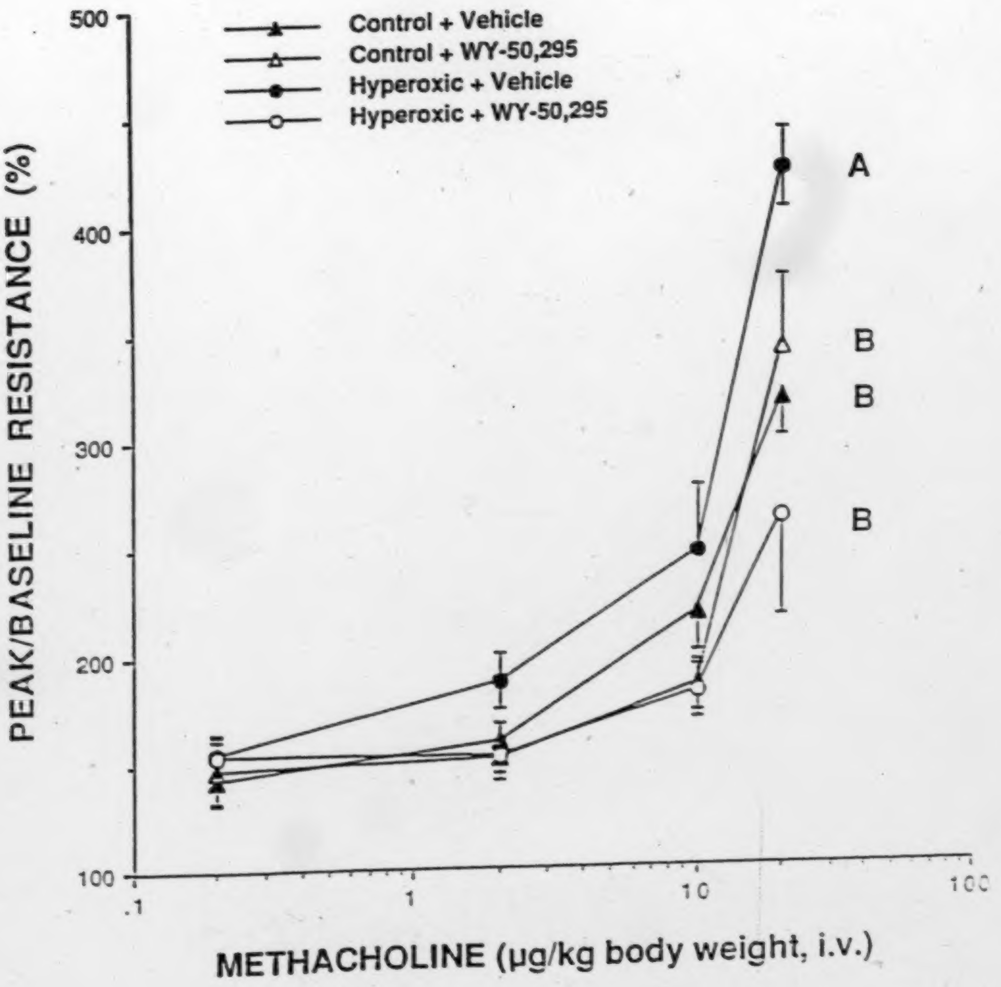


Figure 16. Effect of >95% O_2 exposure and Wy-50295 (30mg/kg daily) on intravenously administered methacholine-induced airway constriction of 5 wk old rat pups. O_2 significantly ($p<0.02$) increased the airway responsiveness to methacholine (n=18) compared with air + vehicle (n=23), air + Wy-50,295 (n=17) and O_2 + Wy-50295 (n=16) groups. Data are expressed as means ± SE. Different letters indicate statistical significance ($p<0.02$) between groups.

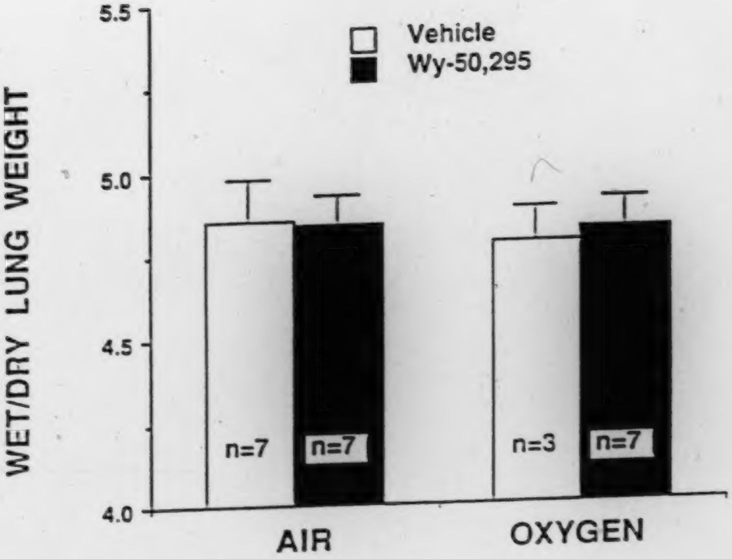


Figure 17. Effects of oxygen exposure and Wy-50295 on wet-to-dry lung weight ratios of 4 wk old rat pups. There were no differences between any of the groups.

Table 9. Effects of O_2 exposure and Wy-50,295 on baseline resistance (BR_L) and EC_{200} values of 5 wk old rat pups.

GROUP	**BR_L cm H_2O/ml/min (n)**	**EC_{200} μg/kg body weight (n)**
Air + Vehicle	0.196 ± 0.083 [a]	9.94 ± 3.45 [a]
	(23)	(23)
Air + Wy-50295	0.197 ± 0.053 [a]	9.14 ± 4.85 [a]
	(17)	(17)
O_2 + Vehicle	0.203 ± 0.062 [a]	5.23 ± 4.37 [b]
	(18)	(18)
O_2 + Wy-50295	0.199 ± 0.057 [a]	11.16 ± 4.26 [a]
	(16)	(16)

EC_{200} is the concentration of methacholine required to raise R_L to 200% of baseline. Values are expressed as means ± SD. Different superscripts = $p<0.05$.

Table 10. Effects of O_2 exposure and Wy=50295 on airway wall morphometry of 5 wk old rat pups.

GROUP	$\frac{A_{EL}\ (\mu m^2)}{L_{BM}\ (\mu m)}$	$\frac{A_{SM}\ (\mu m^2)}{L_{BM}\ (\mu m)}$	$\frac{A_{TW}\ (\mu m^2)}{L_{BM}\ (\mu m)}$
Air + Vehicle	0.684 ± 0.026 [a]	0.432 ± 0.061 [a]	1.116 ± 0.084 [a]
Air + Wy-50295	0.650 ± 0.027 [a]	0.380 ± 0.071 [a]	1.030 ± 0.095 [a]
O_2 + Vehicle	0.697 ± 0.027 [a]	0.691 ± 0.106 [b]	1.388 ± 0.095 [b]
O_2 + Wy-50295	0.669 ± 0.078 [a]	0.481 ± 0.092 [a]	1.166 ± 0.159 [a]

Values are expressed at means of 7-11 airways/rat for 4 rats ± A_{EL}, epithelial layer; A_{SM}, smooth muscle layer area; A_{TW}, total wall area (A_{EL} + A_{SM}); L_{BM}, epithelial basement membrane length. Different superscripts = $p<0.05$.

D. FURTHER INVESTIGATIONS

Having established a role for LTs in newborn hyperoxic lung damage, further studies were initiated to look into how LT levels are increased by hyperoxia, and how they alter alveolarization. The number of replications in some groups is small and therefore these studies are preliminary and are only indicative of possible trends and not firm conclusions.

Immunocytochemistry was used to investigate where 5-LO was located in the lung and if this was different in hyperoxic vs. normoxic rats. In an attempt to discern the roles of individual LTs, the effects of administration of MK-0571 (a LTD_4 receptor antagonist) to hyperoxic rats and administration of LTC_4 and LTD_4 to normal rats on lung development were investigated. Results of these preliminary studies follow.

i. Determining the Location of 5-LO in Newborn Rat Lungs

Objectives:

1) To determine the location of 5-LO in the lungs of normal and oxygen-exposed rat pups and adults.

Hypothesis:

1) Hyperoxia will increase 5-LO amounts in the lungs of newborn rat pups.

Animals:

Group	Oxygen Exposure
Air day 14	21% O_2 from birth
O_2 day 14	>95% O_2 from d4-14

Adult (42d)	21% O_2 from birth

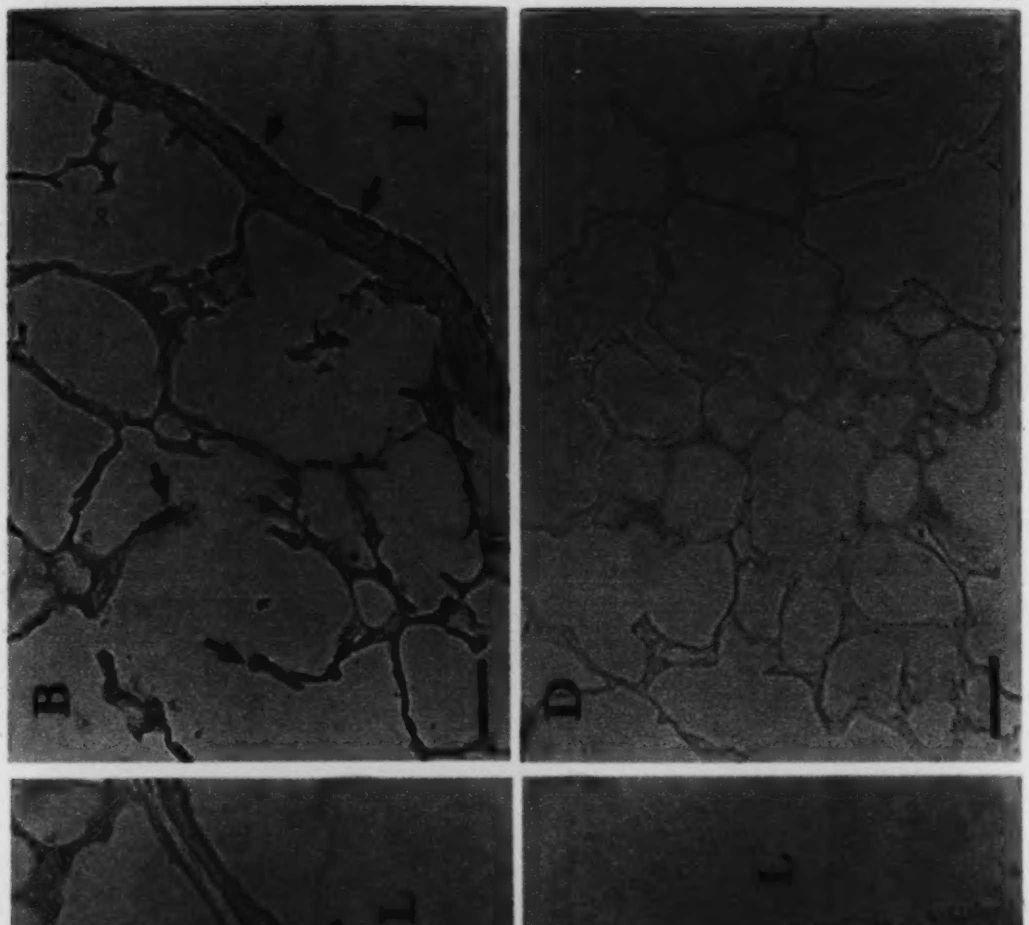
Measurements:

Staining done using polyclonal primary antibodies to 5-LO on sections from lungs of rat pups aged 14 and 42 days.

Results:

Figure 18 shows the sections of lung of 14 day old rat pups exposed to air (A), O_2 (B) and the lung of adult rat (C) stained for 5-LO. Staining for the enzyme shows up as a brownish red color. Figure 18 D is the negative control (14 day old rat lung without primary antibody). Each slide was processed for the same amount of time and is shown at the same magnificantion. What is noticeable from this figure is the relative lack of staining in adults compared to newborn rats. Also, the lungs of hyperoxic rat pups at day 14 show more intense staining for 5-LO than do air exposed rat pups. This is particularly evident in certain airway epithelial cells, as well as various parenchymal cells.

Figure 18. Immunocytochemical staining of 14d old (A and B) and adult (C) rat lungs with a primary antibody to the 5-lipoxygenase enzyme (brownish-red colored stain, arrows). **A**: 21% O_2. **B**: >95% O_2 from d4-14. **C**: Adult. **D**: 21% O_2 negative control (no primary antibody added). L=airway lumen. Staining can be seen to some extent in both 14d old lungs (A and B, arrows), While it is barely evident in air exposed animals (A), it is much more intense after 10d of hyperoxic exposure (B). Both 14d old lungs (A and B) have more intense staining than the adult (C). Bar=40μm.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ii. Effect of LTD_4 Receptor Antagonist, MK-571

Objectives:

1) To determine the effect of LTD_4 receptor antagonism on hyperoxia-induced lung damage in newborn rats.

Hypothesis:

1) If LTD_4 receptors are involved in hyperoxic lung damage, MK-571 will prevent hyperoxia-induced lung damage.

Animals:

Groups	Oxygen Exposure	LT Inhibition
RA + V	21% from birth to day 14	Vehicle (H_2O) sc every 12 hours, days 3-14
RA + MK-0571	21% from birth to day 14	MK-0571 20 mg/kg, sc every 12 hours, days 3-14
O_2 + V	>95% from days 4-14	Vehicle (H_2O) sc every 12 hours, days 3-14
O_2 + MK-0571	>95% from days 4-14	MK-0571 20 mg/kg, sc every 12 hours, days 3-14

Measurements:

Parenchymal morphometry day 14

Results:

Comparative lung morphometric findings for 14 day old rat pups administered MK-0571 (10, 20, 30, or 40 mg/kg, sc, once daily) from days 3-14 while exposed to air or hyperoxia (days 4-14) is shown in Table 11. For simplicity, statistical significance is only shown compared to RA + V or O_2 + V groups (and not between air + MK-0571 or O_2 + MK-0571 groups). The volume density of parenchymal tissue (V_p) of O_2 + V pups was significantly lower than that of RA + V pups. Administration of MK-0571 did not signficantly increase V_p in hyperoxic pups, although it was higher in pups administered 30mg/kg MK-0571 compared to O_2 + V animals. Alveolar septa were not significantly different between any of the groups, however, overall, hyperoxic animals had slightly thicker septa. As with all previous studies, hyperoxia has a significant enlarging effect on alveoli as indicated by increased airspace unit volume ($V_{aspunit}$) compared to RA + V animals. The $V_{aspunit}$ was not signficiantly different in hyperoxia-exposed pups at doses 10-30 mg/kg MK-0571 compared to RA + V animals. These groups were significantly smaller than the O_2 + V group. Hyperoxia also caused a significant decrease in P/A ratio compared to all air groups. While the ratios were higher in pup administered 10-30 mg/kg MK-0571 compared to O_2 + V animals, they were not significantly higher. Nor were they significantly lower than the air groups. While no groups were found to be significantly different from one another in terms of SISA, the trend was towards a lowering due to hyperoxia, which was increased by administration of MK-0571.

Table 11. The effects of hyperoxia and MK-0571 (10-40mg/kg, sc, once daily, d3-14) on lung morphometry of 14 day old rat pups.

GROUP	n	$V_{asp\ unit}$ ($\mu m^3 x10^5$)	P/A	Vp (%)	Th sept (μm)	SISA ($cm^2/gx10^3$)
Air + Vehicle	11	0.54±0.09 Δ	1.03±0.08 Δ	29.8±2.6 Δ	0.81±0.09	5.51±0.80
Air + MK0571(10)	2	0.59±0.01 Δ	1.10±0.03 Δ	28.3±1.4	0.75±0.04	5.11±0.37
Air + MK0571(20)	2	0.61±0.14 Δ	1.08±0.04 Δ	28.3±2.4	0.79±0.02	5.74±0.52
Air + MK0571(30)	5	0.45±0.08 Δ	1.05±0.10 Δ	28.5±1.6	0.74±0.04	5.87±1.40
Air + MK0571(40)	3	0.84±0.10 Δ	1.00±0.11 Δ	28.7±2.2	0.79±0.06	5.24±0.49
O_2 + Vehicle	13	0.92±0.25 *	0.83±0.10 *	25.9±2.6 *	0.78±0.09	4.71±1.00
O_2 + MK0571(10)	2	1.32±0.43 Δ*	0.89±0.13	25.0±3.4	0.81±0.12	4.51±0.14
O_2 + MK0571(20)	2	0.64±0.04 Δ	0.91±0.09	27.2±2.0	0.83±0.09	5.02±1.00
O_2 + MK0571(30)	4	0.72±0.21 Δ	0.93±0.07	31.2±3.0 Δ	0.85±0.07	5.62±1.30
O_2 + MK0571(40)	2	1.19±0.13 *	0.80±0.05 *	23.5±1.8 *	0.78±0.05	4.93±0.17

Values are expressed as means±SD. Δ=$p<0.05$ compared to O_2+Vehicle group. *= $p<0.05$ compared to Air+Vehicle group. $V_{asp\ unit}$, mean airspace unit volume; P/A, ratio of airspace perimeter to area; Vp, volume density of parenchymal tissue; Th sept, mean septal thickness; SISA, specific internal surface area.

iii. Administration of LTC_4 and LTD_4

Objectives:

1) To determine the effects of leukotrienes on the developing lung.

Hypothesis:

1) LTD_4 or LTC_4 administration will prevent alveolarization.

Animals:

Group	Dose
LTD_4	1.0 mg/kg sc 2x daily
LTC_4	1.0 mg/kg sc 2x daily

Results:

Table 12 shows the morphometric data of animals subcutaneously administered 0.1 µg/kg LTD_4, LTC_4, or saline vehicle twice daily from days 4-14. Highly noticeable is that neither LTC_4 nor LTD_4 cause a significant increase in $V_{aspunit}$. In fact there is a significant decrease ($p<0.05$) caused by LTD_4 compared to vehicle controls. Administration of LTD_4 also caused a significant decrease in septal thickness compared to the vehicle group. LTC_4 also caused a significant decrease in septal thickness compared to the vehicle group. SISA was decreased compared to the LTD_4 group by not the vehicle group. At the same time, SISA was not significantly different in animals given LTD_4 compared to controls. There were no significant differences in the P/A between any of the groups.

Table 12. Effects of administration of LTC_4 or LTD_4 (0.1mg/kg, sc, once daily from d4-14) on lung morphometry of 14 day old rats.

GROUP	n	$V_{asp\ unit}$ ($\mu m^3 x 10^5$)	P/A	Vp (%)	Th sept (μm)	SISA ($cm^2/g x 10^3$)
Vehicle	3	0.55±0.01 ^{a}	1.21±0.27 ^{a}	27.0±0.1 ^{a}	0.76±0.01 ^{a}	6.2±0.01 ^{ab}
LTC_4	3	0.67±0.04 ^{a}	1.16±0.02 ^{a}	21.9±0.1 ^{b}	0.64±0.03 ^{b}	5.8±0.1 ^{a}
LTD_4	3	0.40±0.06 ^{b}	0.91±0.07 ^{a}	26.1±0.2 ^{a}	0.64±0.03 ^{b}	6.4±0.04 ^{b}

Values are expressed as means±SD. Different superscripts indicate significant differences at $p<0.05$. $V_{asp\ unit}$, mean airspace unit volume; P/A, ratio of airspace perimeter to area; Vp, volume density of parenchymal tissue; Th sept, mean septal thickness; SISA, specific internal surface area.

iv. The Effects of Hyperoxic Exposure from days 4-14 on Morphometry of 42 day old Rats

Objective:

1) To investigate the long-term consequences of hyperoxic exposure of rat pups from days 4-14 on parenchymal morphometry measured on day 42.

Hypothesis:

1) Hyperoxic exposure from days 4-14 will prevent alveolarization in rat pups that will still be measurable at day 42.

Animals:

Group	Oxygen Exposure
Air	21% O_2 from birth to d 42
O_2	>95% O_2 from d 4-14

Measurements:

Parenchymal morphometry day 42

Results:

Table 13 shows lung morphometric findings for 42 day old rats that had been exposed to 21% from birth to day 42 or >95% O_2 from days 4-14. O_2 animals show a significant decrease in V_p compared to air controls. There was no difference in the thickness of the septa at this age. O_2 animals had significantly higher $V_{asp\ unit}$ compared to air animals and, although not statistically significant, P/A was smaller in these pups. The same trend was evident for SISA, which was lower in O_2 animals but lacking statistical significance.

Table 13. The effects of hyperoxic exposure from d4-14 on lung morphometry of 42d old rats.

GROUP	n	$V_{asp\ unit}$ ($\mu m^3 x 10^5$)	P/A	Vp (%)	Th sept (μm)	SISA ($cm^2/g x 10^3$)
Air +Vehicle	2	0.46±0.05 ^{a}	1.01±0.02 ^{a}	25.5±1.3 ^{a}	0.68±0.06 ^{a}	3.15±0.38 ^{a}
O_2 + Vehicle	2	1.67±0.85 ^{b}	0.72±0.14 ^{a}	19.2±4.3 ^{b}	0.67±0.05 ^{a}	2.35±0.21 ^{a}

Values are expressed as means±SD. Different superscripts indicate significant differences at $p<0.05$. $V_{asp\ unit}$, mean airspace unit volume; P/A, ratio of airspace perimeter to area; Vp, volume density of parenchymal tissue; Th sept, mean septal thickness; SISA, specific internal surface area.

V. DISCUSSION

A. EFFECTS OF HYPEROXIA AND LEUKOTRIENES ON ALVEOLARIZATION

Hyperoxic exposure upsets the normal cellular oxidant-antioxidant defense equilibrium by producing marked increases in O_2 free radical production (321). Prolonged exposure of newborn and adult rats to high levels of O_2 causes lung damage characterized by interstitial and intra-alveolar edema, followed by infiltration of protein, entry of cells, and finally hemorrhage into the alveolar space (56). Inhibition of the process of alveolarization is also a hallmark of hyperoxic exposure during the newborn period (42, 196, 312).

Hyperoxia has a major impact on lung development. It slows lung maturation and causes reduction in alveolar number and surface area by permanently inhibiting the process of septation (196). Saccule septation is permanently diminished and the parenchymal airspace enlarged with irregular dilatation of alveoli and alveolar ducts (285), which exists until adulthood in rats (245). Decreased cell proliferation is a well known concomittant of hyperoxic exposure in the newborn lung, however, certain cell types, including alveolar type II cells, proliferate when exposed to high levels of O_2 (70). Because the process of septation undoubtedly involves a very coordinated proliferation and perhaps apoptosis of specific cell types, a factor that can inhibit DNA synthesis or induce gene expression such as hyperoxia, can easily disrupt this developmental process of the lung (100).

In our newborn rat model, 10 days of exposure to >95% O_2 caused marked interstitial and intra-alveolar edema and proteinosis. Five days of hyperoxia from days 4-9 or 9-14 was associated with edema at day 9 and day 14 respectively. Altered alveolarization occurred in hyperoxic animals leading to increases in airspace unit volume, which corresponds to the results of investigators cited above. This increase in airspace unit volume was maintained until day 42, even though the rats had been removed from O_2 four weeks previous. Thus, we have a good model of newborn hyperoxic lung damage that has allowed me to identify potential mediators involved.

The timing of exposure to O_2 is very important. Hyperoxic exposure after the lung has gone through its developmental phase has different effects than exposure during the period of alveolarization (53, 153, 285). In fact, in the rat, the period of alveolarization (postnatal days 4-14) has been defined as a "critical period" of lung development, during which time any "stresses" to normal development, including O_2, can have profound, long-lasting effects (199).

Hyperoxic exposure at different time periods during alveolarization was investigated in this study as a means of further describing how hyperoxia affects the developing lung. Exposure to >95% O_2 from days 4-9 caused more profound changes in alveolarization (i.e. larger airspace unit volume, smaller P/A etc.) measured on day 14 than did exposure from days 9-14 or 1-4. However, exposure during these latter mentioned time periods had some impact. Exposure from days 9-14 lead to significant septal thinning, while animals exposed from days 1-4 had thicker septa at day 14. Thus, the effects of hyperoxic exposure are highly dependent on the timing of the exposure.

Several investigators have suggested that the eicosanoids produced by inflammatory cells, or by cells lining the alveoli, may be involved in hyperoxic lung damage. Koyanagi (167) showed that exposure to hyperoxia from day 1 leads to marked increases in production of LTB_4 by the lung. Taniguchi (282) found an increase in LTB_4 levels in the bronchoalveolar lavage (BAL) of adult rats exposed to 85% O_2 for 60 hours. Lipoxygenase inhibited animals had reduced mortality. Several investigators have detected increased LT levels due to oxidative stress. Burghuber *et al.* (44) demonstrated increased 5-hydroxyeicosatetranenoic acid production and edema of perfused isolated rat lung caused by glucose oxidase that was inhibited by leukotriene inhibitors. Similarly, increased LTD_4 and LTB_4 were shown in isolated rabbit lung infused with tertiary butyl peroxide. The accompanied increased vascular permeability was amelioarated by the LT antagonist, FPL-55712 (120). Elevated LT concentrations have been found in the BAL fluid of infants with bronchopulmonary dysplasia (BPD) (119, 279). LTB_4 is a well-known chemoattractant for neutrophils, and the activated inflammatory cells can release other radicals and mediators that may damage normal tissue (282). It is known that LTC_4 and LTD_4 increase vascular permeability, and it has been shown that LTs can provoke pressor responses and edema in perfused rat lungs (74, 129)

In our newborn rat model, exposure to >95% O_2 from days 4-14 caused peptido-LT output of lung explants to increase, measured on day 9 or day 14. We were able to show that hyperoxia caused an increase in peptido-LT production by the lung during alveolarization, suggesting that these mediators could influence events occurring during this time period. The widely held idea is that an increase in LTs caused by hyperoxia is due to liberation of arachidonic acid from damaged cell membranes that becomes available for 5-LO. However, the possibility exists that hyperoxia may upregulate the expression of 5-LO or FLAP in the lungs of the developing animal. Immunocytochemical investigation into the location of 5-LO in the lungs indicated that the amount of 5-LO was higher in the hyperoxic lung at day 14 compared to air exposed lungs. Because ICC is not quantitative, any comments on the amounts of the enzyme present must be made with caution. As well, staining for 5-LO was very light in air animals, who presumably should have 5-LO present since they do make LTs. Despite this, it is still evident that the intensity of the staining is markedly increased in hyperoxic animals compared to air animals.

Up to this time, there have been no investigations into the role of LTs in altering parenchymal development. In this study, two LT synthesis inhibitors with differing modes of action, and one LTD_4 receptor antagonist were used to study the possible LT effects. In the study comparing O_2 + Wy-50,295 with the O_2 + Vehicle group, it was evident that the Wy-50,295 group had a more advanced septation process, indicating that LT inhibition has a beneficial effect. Wy-50,295 also caused significant septal thickening measured on day 14 in both the O_2 + Wy-50,295 and RA + Wy-50,295 groups. The bulkiest septa were detected in the O_2 + Wy-50,295 group, possibly due to the combined effect of O_2 exposure and drug administration. The effect of Wy-50,295 on air-exposed animals, despite non-significant inhibition of LTs suggests that Wy-50,295 had an action of the lungs that was not related to 5-LO inhibition. This effect may account for the prevention of damage in the O_2-exposed animals rather that the actual inhibition of LT during the exposure.

Although LTs were significantly inhibited by both Wy-50,295 and MK-0591, we could not detect any influence of either drug on vascular permeability on day 14, and their administration failed to prevent the development of O_2-induced pulmonary edema. O_2 radicals themselves may therefore be the major pathogenic factor in this aspect of hyperoxia-induced lung disease, because of their detrimental effects on endothelial and epithelial barriers.

In this study, MK-0591 was able to prevent the damage to alveolarization caused by O_2 exposure from days 4-14. Essentially, the O_2 + MK-0591 group was indistinguishable from both RA groups. MK-0591 caused no detectable change in the RA group, despite its ability to inhibit the production of peptido-LTs in these animals. The evidence suggests that any protection offered by MK-0591 to the O_2 exposed group is most likely because of the inhibition of LT.

The discovery of a "critical period" for O_2 within the period of septation in the rat lead to the speculation that a "critical period" for the actions of LTs may coincide with this time period. Animals exposed to >95% O_2 from days 4-9 or days 9-14 showed increased production of peptido-LTs in the lung at day 9 and day 14 respectively. These levels returned to normal by day 14 in animals exposed from days 4-9. Exposure to hyperoxia from days 9-14 did not lead to as severe inhibition of alveolarization as pups exposed from days 4-14 or days 4-9, leading to the speculation that days 4-9 may be a critical period for LT actions on alveolarization. MK-0591 was used to investigate this possibility. Animals exposed to O_2 from days 4-14 who were administered MK-0591 from days 3-14, 3-9, or 9-14 all showed relatively normal alveoli on day 14. Thus, LT inhibition at any time during hyperoxic exposure prevented the damage to alveolarization. There was no apparent "critical period" for LTs, but these experiments raised the possibility that hyperoxia-induced inhibition of alveolarization proceeds through an indirect pathway and a cascade of events may have been initiated. LT inhibition during the first half of alveolarization may not allow the cascade to start at the right time, whereas LT inhibition during the second half may not let it continue.

Studies using the LTD_4 receptor antagonist, MK-0571, were also performed. While the number of replications for certain groups in this study were low, it does give us some useful preliminary information. At a dose of 20 or 30 mg/kg , MK-0571 was able to prevent hyperoxia-inhibited alveolarization much the same way as the LT synthesis inhibitors, providing more evidence for the involvement of LTs, specifically the peptido-LTs.

Classical studies of hormone involvement in any system usually endeavor to show three things: 1) that a correlation exists between higher levels of the substance and the process of interest; 2) that inhibition of the substance prevents the process from occurring; and 3) that adding the substance causes the process to occur. So far I have mentioned numbers one and two in terms of investigating the role of LTs in hyperoxia-induced lung disease. It was in the interest of number three that an attempt was made to administer LTC_4 and LTD_4 to normal rat pups from days 4-14 to examine the effect this would have on alveolarization.

Administration of LTC_4 and LTD_4 (0.1 μg/kg, sc, 2 times daily) did not induce edema but did exert some influence on the lung. What is most notable is the finding that LTC_4 was able to significantly increase the airspace unit volume. However, it was not an increase comparable to that seen in hyperoxic animals. LTD_4 caused a decrease in airspace unit volume, the reasons for which are unknown. It is interesting to note that both LTs caused a significant thinning of septa, while a decrease in percent tissue density was only seen in animals exposed to LTC_4. These findings suggest a role for LTC_4 more so than for LTD_4. This may seem contrary to the idea that the LTD_4 receptor antagonist, MK-0571, prevented the damage caused by hyperoxia, however, we must keep in mind the fact that LTC_4 has been shown to activate LTD_4 receptors, and that LTC_4 is metabolized in the lung to LTD_4 and eventually to LTE_4.

Hyperoxia has the ability to influence the expression of numerous factors. In fact, some of these factors are known to affect the levels of LTs and vice versa. For example, hyperoxia can increase the amounts of the transcription factor NFκB (265) which has also been shown to be increased by LTs (177). Hyperoxic exposure has also been shown to increase the transcription factors c-fos, c-jun and AP-1 (of which c-fos and c-jun are a part) which have seen shown to increase the activation of mitogen-activated protein kinase. LTs have also been shown to increase MAPK levels in THP-1 (monocyte-like) cells (26). Hydrogen peroxide also has the capability to increase LTs through an increase in JNK (9). Tumor necrosis factor (TNF) levels are also increased in hyperoxia. Many TNF responses involve increased rate of transcription of target genes, often through the activation of NFκB or AP-1. Regulation of AP-1 activity is mediated by a stress-activated protein kinase cascade, involving c-jun N-terminal kinase. TNF acts locally as a paracrine and autocrine regulator of leukocytes and endothelial cells and has the ability, like interleukin-1 (IL-1, also an inflammatory mediator), to increase LT levels (224). It is interesting that in adipogenic cells, cell killing induced by TNFα occurs via a pathway in which lipoxygenase products directly or indirectly operate (52). Other cytokines such as

GM-CSF and IL-3 also increase the expression of 5-LO and FLAP and may also be involved (39, 250).

Evidence exists to suggest growth factors as mediators of LT actions on alveolarization. The question remains as to whether or not the effects of LTs are direct, or mediated by the growth factors which have obvious roles to play in lung development; or whether the roles of growth factors could be mediated by LTs. Most studies to this effect have been performed on adult animals. The effects of hyperoxia in an animal in which the bulk of development has already ended, is much different compared to newborn animals in which development is still occurring. Likewise, the role of growth factors in the newborn period is likely different than during adulthood. Keratinocyte growth factor (KGF), and epithelial cell growth factor with mitogenic activity for adult alveolar type II cells *in vitro* (227) and *in vivo* (291). KGF has been shown to protect adult male rats from hyperoxic and other forms of oxidant lung injury (228, 314). Sporn *et al.*(275) showed that KGF enhances alveolar type II cell synthesis of LTC_4 and suggested that in adults alveolar type II cells may be an important source of pro-inflammatory LTs in the setting of lung injury, but that it is also possible, in adult animals, that LTC_4 may be important in epithelial cell repair. Platelet derived growth factor (PDGF) is another growth factor that is known to increase the levels of LTs which have been shown to mediate the mitogenic effects of this growth factor as well, through the activation of c-fos (26). While inhibition of alveolarization may be thought of as a cessation of cell processes, there may be certain cell types that proliferate. Evidence has shown that premature septal thinning due to the premature development of a "single capillary network" as opposed to a "double network", may contribute to the lack of alveolarization

(290). This process undoubtedly involves steps involved in angiogenesis including: 1) degradation of basement membrane; 2) invasion/migration of endothelial cells; 3) proliferation; 4) reestablishment of basement membrane, in which growth factors including PDGF, epidermal growth factor (EGF) and fibroblast growth factor (FGF) are major factors. EGF and FGF have been shown to increase with hyperoxic exposure (139, 220).

Septal thinning may be indicated as a factor which may have a major role in inhibition of septation by LTs. Tschanz *et al.* (290) found that premature septal thinning may be the reason glucocorticoids prevent septation, and that if premature septal thinning occurs before septa erupt, septation will not occur (i.e. for septa to form there must be a double capillary network). Glucocorticoids have been shown to regulate the amount of 5-LO and FLAP in monocytic cells (116, 249). In fact, dexamethasone was shown to increase the expression of 5-LO and FLAP. Along with the findings from this study that hyperoxia from days 4-9 or days 9-14 caused septal thinning but that this only translated into inhibition of alveolarization in the group in which this occurred at the earliest time, and the indication that administration of LTC_4 and LTD_4 caused septal thinning in normal rats, this information points to premature septal thinning as a possible mechanism of LT-inhibited alveolarization.

There is a possibility that other mediators could be responsible for the changes seen. Wy-50,295 also inhibits platelet activating factor (PAF) production by rat peritoneal mast cells (140), and PAF has many actions that mimic those of peptido-LT (edema, mucus production, bronchoconstriction, hyperresponsiveness). Whether hyperoxia increases PAF levels is uncertain because prolonged exposure of adult guinea pigs to hyperbaric O_2 causes an increase in lyso-PAF, an inactive metabolite of PAF, while not affecting the levels of PAF itself (230). Furthermore, Wy-50,295 is 12-fold more effective as an LT inhibitor than a PAF synthesis inhibitor (140, 214), and LT output from lung tissue correlates with the changes we have seen in lung development. However, because Wy-50,295 might have been inhibiting PAF production or have other unknown effects on the lung, further experiments using a more specific LT synthesis inhibitor were necessary.

The possibility of a shunt of arachidonic acid to the cyclooxygenase pathway due to inhibition of 5-LO exists. Nagai *et al.* (216) concluded that normal alveolar formation in rats from days 4-14 depends on the presence of PGs since animals administered indomethacin had altered alveolar development. Also, animals receiving PG and indomethacin at the same time showed fewer abnormalities. However, they did not measure the LT production of the indomethacin-administered groups, and, therefore, the possibility of an arachidonic shunt to the 5-LO pathway was not excluded. We did not detect an elevation of 6-keto $PGF_{1\alpha}$ production at day 14 in the Wy-50,295 or MK-0591 treated animals. We detected high 6-keto-$PGF_{1\alpha}$ levels in all hyperoxic groups due to O_2 exposure. Hageman *et al.* (121) found a similar effect in mature rabbits after O_2 exposure. Hence it is unlikely that the effects observed in the O_2- or drug-treated groups on alveolar development could be caused by altered PG levels.

B. EFFECTS OF HYPEROXIA AND LEUKOTRIENES ON NEWBORN AIRWAYS

Hyperoxic exposure can lead to marked changes in the airways of the lungs, leading to increased airway smooth muscle and changes in airway epithelial cells (134, 293). In animal studies of acute, relatively short term exposures, newborns exposed to high levels of O_2 develop increased airway reactivity. In an acute exposure (85% O_2 for 84 hours), 1-2 day old guinea pigs exhibited increased airway reactivity to acetylcholine without changes in airway histology (293). In contrast, my study of sustained or chronic exposure had newborn pups exposed to >95% O_2 from days 4-14 and 65% O_2 from days 14–32. Pulmonary function and morphometric measurements were obtained from animals 2 days after the cessation of hyperoxic exposure. This was done in an attempt to investigate the chronic effects of O_2, as opposed to its acute effects.

The methacholine dose-response curve for O_2+ Vehicle rats was shifted to the left compared with that of air animals, indicating the development of airway hyperresponsiveness. The lower EC_{200} values for the O_2 + Vehicle group confirmed this. Methacholine provocation of airways was carried out only with intravenous administration of the agent and not combined with methacholine aerosol challenge. The distribution of the contractile agonist during intravenous and aerosol administration is likely to be different. It was assumed that the distribution of agonist administered intravenously would be relatively homogeneous throughout the lung and therefore appropriate for small airway challenge. Nagase *et al.* (217) concluded that the intravenous administration of methacholine induced more modest, but relatively homogeneous constriction of the bronchial tree and lesser degree of tissue distortion. Meanwhile, aerosol administration had a heterogeneous effect, and less airway constriction was observed as airways decreased in size. Because large airways proximal to airway generation 14 (313) with outer circumferences greater than 500 μm were not analyzed in the morphometric study, methacholine challenge of small airways was more relevant. The absence of edema in 4 week old rats, as indicated by similar wet/dry lung weight ratios and similar epithelial layer thickness in oxygen- and air-exposed animals, eliminated the possibility that increased R_L was due to airway obstruction caused by increased vascular permeability.

One of the possible causes for increased airway responsiveness is airway smooth muscle thickening. This phenomenon is observed in patients with BPD (191). Morphometric analysis of small airways (<500 µm in circumference) in this study showed some striking differences between the O_2 + Vehicle group and the air group. Hyperoxic exposure caused a significant thickening of the smooth muscle layer area. Whether this is due to hypertrophy or to hyperplasia of smooth muscle cells or an increase in collagen is unknown. Change in the amount of airway smooth muscle is a plausible explanation for increases in airway reactivity induced by hyperoxic exposure (134).

Hershenson *et al.* (134) found that exposing 21 day old rats to >95% O_2 for 8 days increased the thickness of the epithelial layer. In this study, no differences were found in airway epithelial thickness. This may stem from the fact that this model is one of long-term hyperoxic exposure, and it is possible that epithelial layer repair occurred before morphometric analysis was accomplished. Such evidence exists in 21 day old rats exposed to >85% O_2 for 8 days where airway repair occurs gradually from the time animals are removed from oxygen. In fact, epithelial cell repair occurs at a much faster rate during the first 3 days after oxygen exposure than repair in the smooth muscle layer which does not change at all during this period (134). It is interesting to note that the epithelial layer of 14 day old O_2 exposed animals appears to be thicker than that of air-exposed animals (ICC, Figure 18). Airway morphometric analysis was not performed on these animals.

In the search for mechanisms to translate the chronic effects of O_2 to altered smooth muscle development and subsequent physiologic changes, it is logical to explore the potential roles of peptido-LTs. As mentioned, they are potent bronchoconstrictors and inducers of mucus secretion and vascular permeability. They are found in high concentrations in the BAL of infants with BPD (208, 279) and in the blood, BAL and urine of asthmatics (131). In our model, hyperoxia caused an increase in the production of peptido-LTs by the lung and large airway explants at 2 weeks, and these levels remained elevated only in the large airways at 4 weeks of age.

Although measuring the large airway explant peptido-LT production can give insight into large airway synthesis, it does not necessarily reflect synthesis by small airways. In an attempt to approach this issue, the output of peptido-LTs from lung parenchymal slices was used as an indication of LTs that could more readily affect small airways. There were indeed differences in peptido-LT output between large airway explants and lung slices, especially at 4 weeks. At 2 weeks, hyperoxia enhanced LT output from each type of preparation. However, at 4 weeks, only the large airways responded to hyperoxia by increasing peptido-LT output. It may be that non-airway tissue of the parenchyma might have a different time course of responsiveness to hyperoxia. Regardless, it is clear that peptido-LT production in response to hyperoxia is enhanced and is prolonged sufficiently to alter pulmonary development.

This study does suggest the possibility that peptido-LTs mediate O_2-altered lung changes. Inhibition of LTs by Wy-50,295 prevents the development of O_2-induced airway hyperresponsiveness and airway smooth muscle layer thickening. Wy-50,295 inhibited peptido-LT production of the large airway of O_2-exposed rats and reduced peptido-LT levels to that of air animals at both 2 and 4 weeks. The levels were significantly lower than O_2 + Vehicle animals, but they were not as low as those in air animals. At 4 weeks, Wy-50,295 significantly reduced peptido-LT production to below normal air values. Generally, Wy-50,295 had no significant effect on peptido-LT production in air animals, except in the lung slices at 4 weeks. Hence, LT output from lung tissue correlates with the changes (or lack of changes) observed.

Little is known about the effects of chronic peptido-LT exposure on airway smooth muscle. Airway smooth muscle has receptors for peptido-LTs, and LTD_4 receptor antagonists have been shown to eliminate LTD_4- induced airway thickening as well as hyperresponsiveness in asthma models (53-55). Rajah *et al.* (243) has shown that LTD_4 is comitogenic with insulin –like growth factors (IGF) on cultured human airway smooth muscle cells. The synergistic effect involved the proteolysis of airway smooth muscle produced inhibitory IGF-binding proteins (IGFBP). LTD_4 induced a reduction in IGFBP by inducing the production of IGFBP protease. Thus, peptido-LTs may work indirectly, with growth factors in airways.

Alternatively, peptido-LTs may cause a chronic contraction of airways leading to hypertrophy. Exposing cultured rat trachea smooth muscle cells to 70% O_2 resulted in increased smooth muscle cell protein content compared with cells exposed to 21% O_2 (2). Smooth muscle cell proliferation was inhibited by O_2 in the same study, suggesting that O_2 induced hypertrophy. Peptido-LTs may play a role in maintaining pathologic tone in asthmatics. Also, human bronchial smooth muscle exhibits a high level of intrinsic tone that is due to continual production and release of peptido-LTs (149). In our study, baseline R_L was higher in the O_2 + Vehicle group than all other groups, but the difference was not statistically significant. Subjective analysis of immunocytochemical localization of 5-LO in 14 day old animals showed the levels of 5-LO to be higher in certain airway epithelial cells compared to air animals. While it is unknown at this point exactly which type of airway epithelial cells they are; this is consistent with other studies which have located 5-LO in non-ciliated bronchiolar epithelial cells in pigs (165). Hence, Clara cells may be a candidate for increased levels of 5-LO in the airway epithelium. Pulmonary neuroendocrine cells may also be a candidate for increased levels of 5-LO and FLAP, as the number and size of these cells increase during hyperoxic exposure (266), however, the amount of staining in the airway epithelium seems to be more frequent than would be indicative of neuroendocrine cells, since these cells occur in a relatively few discreet bundles.

C. FUTURE STUDIES

While these investigations provide evidence linking hyperoxia-induced lung damage to LTs, many questions remain to be answered. Some of these questions and possible ways of addressing them are outlined below.

1) Hyperoxia exposure from days 4-9 results in inhibition of alveolarization, but are these changes permanent? Do they last beyond 14 days? Alternatively, were animals exposed to O_2 from days 9-14 given long enough time to manifest changes? Whereas animals exposed to hyperoxia from days 4-9 had an extra 5 days before morphometry on day 14,

pups exposed to hyperoxia from days 9-14 were measured right at the end of the exposure period. Both of these questions could be investigated by exposing the animals as before, but measuring the morphometry at later time points.

2) Which exact cell types are producing LTs? Light level microscopy of lung slices stained for 5-LO indicate increased levels with hyperoxia. However, studies should be undertaken to examine exactly which cell types are involved. Using markers for specific cell types or electron microscopy could address this question. ICC studies should also be performed looking at FLAP.

3) How is hyperoxia altering the levels of leukotrienes? More detailed studies looking at the expression of 5-LO and FLAP in hyperoxic animals would be an interesting starting point to answer this question. Western blots could be performed to determine the relative amounts of the proteins present. If we can determine how hyperoxia alters the production of LTs, for example, by changes in expression of 5-LO and FLAP, and if we can determine when these changes occur, we can get more clues as to which stages are important in normal lung development.

4) What process is hyperoxia altering at days 1-4 to get increased septal thickening at d14? Looking at the development microvasculature of the lungs may be helpful in answering this question.

5) Which LTs are actually important? In this study, LTB_4 was not the major focus, however, when 5-LO is inhibited, presumably, the synthesis of LTB_4 and peptido-LTs is prevented. Studies administering the various LTs, perhaps with a long-term pellet implant type device could be performed, this time monitoring the levels in the animal and examining the metabolism of the LTs. As well, it would be beneficial to repeat the LTD_4 receptor antagonist studies with a better inhibitor.

6) Perhaps the biggest question coming from this study is how are LTs mediating changes in the development of the lung? Are they working directly on cells involved in alveolarization or are they initiating a cascade of events involving numerous mediators? Determining the sites of receptors for LTs could indicate where LTs are acting. LTs may be acting as second messengers for many growth factor mediated events. To study this, the levels of various growth factors at different times during hyperoxia could be investigated. It might be determined whether inhibition of LT synthesis results in a decrease or increase of these growth factors.

D. CONCLUSIONS

While much is known about the acute effects of LTs in adult models of lung disease (especially as it pertains to asthma and airway disease), before this study, little was known about the role LTs played in the manifestation of newborn lung disease. Changing the levels of any factor during a period of heightened development (eg. alveolarization) is likely to alter that process. Because the development of the airways is mostly complete by this time in rats, it is likely that the effects of LTs and hyperoxia on airway cells is different that on cells involved in septation. In fact, it would not be too "far fetched" to speculate that the same factor may have differing types of effects in the two areas of the lung. While previous studies have shown an increase in LT levels due to hyperoxia or BPD, this is the first time inhibition of LTs has be shown to prevent hyperoxic-induced alveolar damage in newborns. With the development of new anti-LT therapies for use in asthma, the implications of this study for the prevention of diseases like BPD becomes obvious. Also important is that, because of this study, LTs have now become another tool for teasing apart the intricacies of how the normal lung develops.

However, having said this, this study also shows that LTs do not work alone during hyperoxia. Certain pathologic changes caused by hyperoxia such as edema formation and proteinosis were unaffected by LT inhibition and administering LTC_4 and LTD_4 alone did not result in the massive changes seen during hyperoxic exposure. It is much more plausible to assume that LTs are involved in a cascade of release of various mediators including cytokines and growth factors. I have found one very small piece in a puzzle that is sure to have millions of pieces.

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